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MODIFICATIONS OF THE FUNCTIONING OF PHOTOSYSTEM II INDUCED IN THE DARK BY ALKALINE pH IN THE PRESENCE OF CATIONS

BRIGITTA MAISON-PETERI, CLAUDIE VERNOTTE and JEAN-MARIE BRIANTAIS
Laboratoire de Photosynthèse, C.N.R.S., 91190 Gif-sur-Yvette (France)

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Submission of chloroplasts to alkaline pH, in the range pH 7.5–9.5, leads to changes in their oxygen-evolving capacities. These changes are enhanced by the addition of divalent cations and also monovalent cations at high concentrations. (1) Dark incubation of chloroplasts at pH ⩾ 9 gives rise to a time-dependent inactivation of electron transport from water to 2,6-dichlorophenolindophenol measured at neutral pH. The rate of inactivation is increased by adding cations. (2) The variable fluorescence is decreased with a dependence on incubation time and concentration of cations similar to that of the Hill reaction. Addition of the electron donor NH_2OH removes most of the fluorescence quenching, (3) EPR measurements indicate that the inactivations are accompanied by loss of Mn^{2+} and the appearance of signal II fast. (4) At lower pH (7.5) the oscillations of oxygen evolved per flash during a sequence of flashes show an increase in damping when 20 mM $MgCl_2$ is present instead of 100 mM KCl. These changes are not seen at pH 6. (5) None of these Mg^{2+}-induced modifications are prevented by glutaraldehyde fixation. We conclude that the effects of alkaline pH and $MgCl_2$ do not involve major protein structural changes, and that both act on the manganese-containing protein of the oxygen-evolving site.

Introduction

It has been known for a long time that the intrathylakoid pH influences the rate of electron transport across Photosystem II [1,2]. An alkaline pH in the presence of uncouplers causes an inactivation of the oxygen-evolving apparatus, but addition of artificial electron donors can restore electron transport.

Illumination of the thylakoids has also been shown to have an influence on this inactivation and it has been proposed [3] that a photoinduced conformational change in Photosystem II makes the water-splitting enzyme more labile at high pH. Briantais et al. [4], using flash illumination, demonstrated that the photoinduced inactivation took place predominantly on the S_2 state, when two positive charges have been accumulated on the water-splitting enzyme.

Cations, especially divalent ones, are also known to affect electron flow through Photosystem II [5–13]. Several authors [9–12] observed a stimulation of Photosystem II activity by 2–10 mM $MgCl_2$. Using much higher concentrations (50–300 mM), Chen and Wang [13] pointed out that Mg^{2+} had a Tris-like effect at pH 7.9 in extracting the manganese from Photosystem II.

In the present study we have considered the influence of cations on the dark inactivation of the Hill reaction at alkaline pH and tried to correlate these effects with our observation that Mg^{2+} increases the degree of damping of oxygen sequences at physiological pH.

Abbreviations: DCIP, 2,6-dichlorophenolindophenol; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; Tricine, *N*-tris(hydroxymethyl)methylglycine; Pipes, 1,4-piperazinediethanesulfonic acid; Chl, chlorophyll.

Materials and Methods

Chloroplast preparation. Broken chloroplasts were isolated from spinach, lettuce or peas, according to a procedure previously described [10] and were resuspended in 0.4 M sucrose, 10 mM NaCl, 10 mM Tricine, pH 7.8. They were stored in the dark at 0°C at a concentration corresponding to 1–3 mg Chl/ml.

Inactivation treatment. Before use, the chloroplasts were diluted to the required concentration in 1 mM Tricine buffer, pH 7.8, containing 0.4 M sucrose, 10 mM NaCl, 10^{-6} M gramicidin D and various concentrations of cations. A small amount of molar glycine-NaOH, pH 10, was injected into the suspension in the dark to bring the pH to a value between 9.0 and 9.5, unless otherwise indicated. Samples were taken at different time intervals and neutralized using molar Tricine, pH 7.0. Alternatively, samples were incubated for a given time, either varying the pH or the cation concentration ($MgCl_2$ from 1 to 100 mM or KCl from 100 to 800 mM).

Activity measurements. The rate at the Hill reaction was measured, always at neutral pH, spectroscopically by the photoinduced changes of DCIP absorbance at 580 nm in a Cary 14 spectrophotometer with saturating light [4]. The chloroplasts were used at a concentration of 10 μg Chl/ml.

Fluorescence. The induction at 685 nm was determined at room temperature either without any additions or in the presence of 10 mM NH_2OH. The oscillations of the initial fluorescence due to the B/B^- couple were followed at pH 7.5, according to the method of Wollman [14] using apparatus previously described [15]. Chloroplasts were incubated at a concentration of 30 μg Chl/ml with either 100 mM KCl or 20 mM $MgCl_2$ in the Tricine buffer. The sample was submitted to the desired number of preilluminating flashes, followed by addition of 10^{-4} M DCMU, and the initial fluorescence yield was measured approx. 0.4 s after the last flash.

EPR. Measurements were performed using a Bruker B-ER 420 spectrometer. Instrument settings were as follows: 6.3 G modulation, 25 mW microwave power for signal II spectra; 16 G and 100 mW for Mn^{2+}. Chloroplasts were used at a concentration of 1 mg Chl/ml in a flat quartz cell. A Phaser flash-pumped laser was used for flash illumination of the EPR cell.

To overcome the quenching effect on the Mn^{2+} signal of the buffers used for the alkalinization we used the following protocol: chloroplasts were diluted to 50 μg Chl and then underwent the same alkaline inactivation procedure as described above. The samples were centrifuged at 1 500 × *g* for 10 min and the pellets resuspended in Hepes buffer, pH 7.5. The samples were each divided into two groups. One group was left untreated and tested for signal II fast and the Hill activities were determined. The other group was heated for 5 min to 70°C and tested for Mn^{2+}.

Oxygen sequences. The yield of oxygen evolved per flash during a series of short saturating flashes was measured with a rate electrode. Chloroplasts were used at a concentration of 500 μg Chl/ml. Buffers used for the circulating medium were 10 mM Pipes, pH 6, or 10 mM Hepes, pH 7.5; either 100 mM KCl or 20 mM $MgCl_2$ was added. The degree of damping was calculated using a programme developed by Lavorel [16].

Glutaraldehyde fixation. Fixation and characterization of fixed membranes were done according to the method of Zilinskas and Govindjee [17]. Chloroplasts (150 μg Chl/ml) in 0.4 M sorbitol, 10 mM NaCl, 10 mM Tricine, pH 7.8, ±10 mM $MgCl_2$, were incubated for 5 min in the dark at 0°C with 0.5% glutaraldehyde. The suspensions were centrifuged and the pellets resuspended in various media in order to perform the following assays: (a) characterization of the fixation (inhibition of chloroplastic osmotic configurational changes [17]); (b) inactivation treatment by alkaline pH; (c) oxygen sequence measurements.

Results

Hill reaction measurements

The inactivation of the Hill reaction from water to DCIP depends on the time of incubation at alkaline pH with apparent first-order kinetics. In Fig. 1a the rates of inactivation are plotted vs. pH with or without 10 mM $MgCl_2$ in addition to 10 or 100 mM NaCl. (No difference was observed between 10 and 100 mM NaCl. These two concentrations were used to detect if the variations might be due to an effect on the spill-over). Fig. 1a was derived from a collection of measurements. Fig. 1b shows the pH dependence of the inactivation for a single experiment

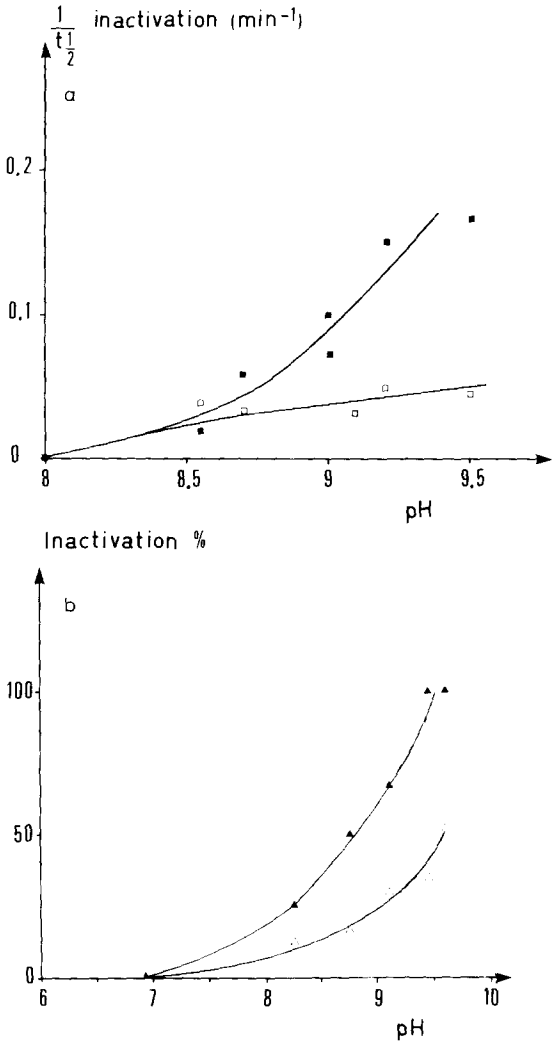

Fig. 1. Dependence of the inactivation on pH. (a) Hill activity at neutral pH of samples incubated for various times at a given pH. The half-times of inactivation are plotted vs. the pH of incubation. (□) Control: chloroplasts were inactivated as described in Materials and Methods with either 10 or 100 mM NaCl present in the medium. (■) 10 mM $MgCl_2$: same as control plus 10 mM $MgCl_2$. (b) Hill activity at neutral pH for samples incubated for 10 min at various pH values. (△) Control: 10 mM NaCl in the medium. (▲) 10 mM $MgCl_2$: same as control plus 10 mM $MgCl_2$.

where chloroplasts were incubated for 10 min at a given pH with or without 10 mM $MgCl_2$.

Table I shows the effects of glutaraldehyde fixation on the inactivation caused by 10 min incubation at pH 9.3. The fixation was carried out either in the presence or in the absence of $MgCl_2$ and equally the

TABLE I

Hill activity (H_2O to DCIP) of control and glutaraldehyde-fixed thylakoids after 10 min incubation at pH 7 or pH 9.3 in relative units. Note that the control underwent the same treatment, but without addition of glutaraldehyde, as the fixed chloroplasts. B, both control and glutaraldehyde-fixed samples were resuspended in a medium containing 10 mM $MgCl_2$, then they were centrifuged and resuspended with or without $MgCl_2$.

	Mg (mM)	Hill activity		% inhibition by alkalinization
		pH 7	pH 9.3	
(A) Control	0	100 [a]	92	8
	10	83	29	65
Glutaraldehyde fixed in the absence of $MgCl_2$	0	15	15	0
	10	25	12	52
(B) Control	0	74	57	23
	10	71	15	79
Glutaraldehyde fixed in the presence of 10 mM $MgCl_2$	0	21	16	22
	10	20	11	45

[a] 100 = 200 μmol DCIP reduced/mg chlorophyll per h.

alkalinization was done in both cases with or without addition of 10 mM $MgCl_2$. The Hill activities after treatment with glutaraldehyde were considerably lower than those of the control, but the glutaraldehyde treatment did not suppress the synergistic action of alkaline pH and Mg^{2+}.

Monovalent cations such as Na^+ and K^+ also stimulate the inactivation of oxygen evolution but much higher concentrations are required. For example, after 5 min incubation at pH 9.3, half inactivation was obtained with 2.5 mM $MgCl_2$ or 330 mM KCl.

We have used fluorescence and EPR measurements to characterize further this inactivation, and verify that it is due to an action on the oxygen-evolving side in accordance with other authors [1–4].

Fluorescence measurements

The initial level of fluorescence was not affected by alkaline incubation. However, the fluorescence

maximum, F_m, was decreased by the alkaline inactivation. The higher the pH and Mg^{2+} concentration, the greater was the fluorescence quenching (Fig. 2). Addition of 10 mM NH_2OH, an electron donor to Photosystem II, to the inactivated material led to the recovery of most of the fluorescence.

EPR measurements

Most treatments which affect the Photosystem II donor side between water and the site of donation of NH_2OH lead to solubilization of Mn^{2+}. Manganese has been shown to play a key role in photosynthetic oxygen evolution [18] and, when liberated from the thylakoid membrane, is detectable by EPR. Concomitant with the liberation of Mn^{2+} is the appearance of a light-induced EPR signal, signal II fast [19].

We have followed the behavior of these two signals for the alkaline-inactivated material. The results obtained are shown in Fig. 3 which depicts a plot of manganese remaining after alkaline inactivation (liberated by the heat treatment) and signal II fast vs. Hill activity. It is obvious from Fig. 3 that 10 min alkaline inactivation in the presence of $MgCl_2$ released more Mn^{2+}, i.e., there was less remaining, and increased the amplitude of signal II fast more than the alkaline treatment alone.

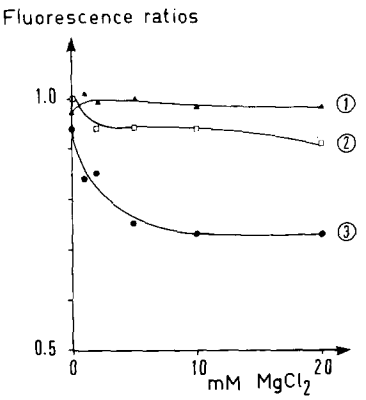

Fig. 2. Chlorophyll *a* fluorescence (F_m) at neutral pH of samples incubated for 5 min at pH 7.3 or 9.3 in the presence of various concentrations of $MgCl_2$. When present, 10 mM NH_2OH was added 5 min before the measurements. The fluorescence level is shown as F_m (NH_2OH). (1) Ratio of F_m (NH_2OH) of samples incubated at pH 9.3 to F_m (NH_2OH) of samples incubated at pH 7.3. (2) Ratio of F_m to F_m (NH_2OH) of samples treated at pH 7.3. (3) Ratio of F_m to F_m (NH_2OH) of samples incubated at pH 9.3.

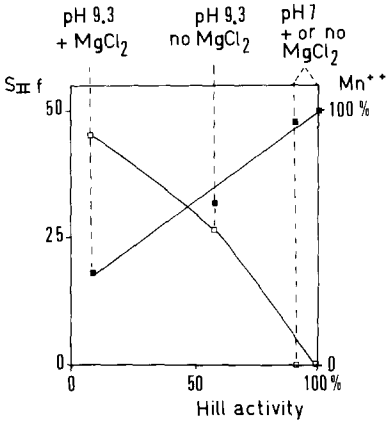

Fig. 3. Relationship between Hill activity, Mn^{2+} content (■) and signal II fast ($S_{II}f$) (□) of samples incubated 10 min at pH 7.3 or 9.3 with or without $MgCl_2$. For details see Materials and Methods.

These results confirm our hypothesis that the enhancement of alkaline inactivation by $MgCl_2$ is due to its action on the oxygen-evolving site in a manner analogous to Tris.

We have tried to see if addition of $MgCl_2$ leads to pH-dependent perturbations of the water-splitting enzyme as detected via the oxygen sequences.

Oxygen sequences

At pH 6 and 7.5 the Hill reaction rates with either 100 mM KCl or 20 mM $MgCl_2$ were equal. In Fig. 4 the oxygen sequences of chloroplasts with either 100 mM KCl or 20 mM $MgCl_2$ as electrolytes are shown for pH 6 and 7.5. At pH 7.5 $MgCl_2$ increased the damping of the oscillations as compared with KCl, whereas at pH 6 there were no differences. We have calculated the degree of damping and found σ values between 0.3 and 0.4 (Fig. 4), except for $MgCl_2$ at pH 7.5 where σ equals 0.7. The latter value is similar to that obtained for *Chlorella* cells.

Table II gives the σ values for oxygen sequences at pH 7.5 with KCl or $MgCl_2$ for unfixed (control) and glutaraldehyde-fixed chloroplasts. Although the fixed membranes had only half the activity of the controls, the same increase in damping was observed on $MgCl_2$ addition in both cases.

The increase in damping could be due to an acceleration of the rates of decay of the S_2 and S_3 states. This would create disorder among the S states and

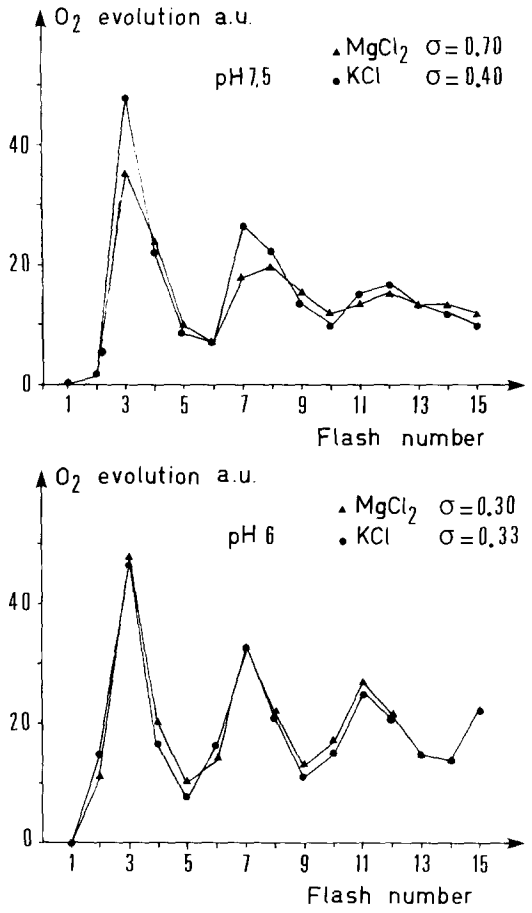

Fig. 4. Oxygen yield as a function of flash number for chloroplasts suspended at pH 6.0 or 7.5 in the presence of 100 mM KCl (•) or 20 mM $MgCl_2$ (▲) as electrolyte. The damping coefficients (σ) were determined according to the method of Lavorel [16]. a.u., arbitrary units.

hence increase the damping. However, we found no significant differences between KCl and $MgCl_2$ for the rates of decay of the S_2 and S_3 states.

We have also tested the effect of $MgCl_2$ on the fluorescence oscillations. If the damping of the latter were changed, as well as those of oxygen evolution, this would imply that the damping seen with $MgCl_2$ is due to an enhancement of a back reaction between the donor and acceptor side.

Binary oscillations of the initial fluorescence yield can be detected in chloroplasts when flash illumination is followed by rapid addition of DCMU [14]. These oscillations result from the fact that the

TABLE II

σ values calculated according to the method of Lavorel [16] from oxygen sequences at pH 7.5, for control and glutaraldehyde-fixed thylakoids. For details see Materials and Methods.

	100 mM KCl	20 mM $MgCl_2$
Control	0.43	0.56
Glutaraldehyde fixed	0.40	0.55

secondary acceptor B needs to accumulate two electrons before transferring them to the plastoquinone pool. Addition of DCMU causes a back transfer of electrons from B to the primary acceptor Q, resulting in a fluorescence level modulated by the redox state of B.

The degree of damping of the oscillations can be calculated in the same way as those of oxygen evolution. Thus, we measured the initial fluorescence levels with either 100 mM KCl or 20 mM $MgCl_2$ at pH 7.5. We found that there was no difference in the damping with either $MgCl_2$ or KCl present.

At pH 7.5, $MgCl_2$ only affected the damping seen from the donor side and would seem to act essentially on the water-splitting enzyme. However, we must take into account that it is not certain whether all centers function via B/B^- [15], and this could influence the detection of the fluorescence oscillations.

Discussion

A number of authors have shown that light-induced inactivation by alkalinization takes place predominantly on the donor side of Photosystem II [1–4] and essentially via the S_2 state. This is similar to the effects observed after Tris treatment [20,21].

Our results show that the presence of $MgCl_2$ enhanced the rate of alkaline inactivation in a manner similar to exposure to light. Equally, monovalent cations are able to induce an increase in inactivation of the Hill reaction but they need to be present in concentrations at least a 100-times higher than those of divalent cations. The effect of $MgCl_2$ is to stimulate the action of alkaline pH on the water-splitting enzyme, as shown by EPR and variable fluorescence measurements. Mg^{2+} produced an enhancement of the

release of manganese and the appearance of signal II fast after treatment at pH > 9. Equally, Mg^{2+} induced supplementary quenching of the variable fluorescence which could be removed by addition of NH_2OH. These effects are similar to those observed for the light enhancement of inactivation via the S_2 state of alkaline or Tris treatments.

The preferential inactivation of the water-splitting enzyme in the S_2 state by alkaline pH [4] or Tris [21] may have several origins: the formation of the S_2 state may induce a conformational change of the enzyme which renders the S complex more sensitive to external treatments. Alternatively, in the S state the link between manganese and the enzyme may be weakened, thus allowing the manganese to be more easily detached.

However, our results do not permit us to say whether the S_2 state is involved, as is the case for the enhancement of inactivation by light. The possibility cannot be excluded that the presence of Mg^{2+} at high pH leads to the formation of some S_2. Unfortunately this is not possible to test.

Frasch and Cheniae [21] have shown that glutaraldehyde fixation protects against flash-induced inactivation by Tris. In contrast, Briantais et al. [4] did not see any effect of glutaraldehyde fixation on the increase of inactivation produced by light at alkaline pH. Comparison of these contradictory results is complicated by the fact that glutaraldehyde fixation was done by Frasch and Cheniae [21] in a medium containing 10 mM $MgCl_2$ and by Briantais et al. [4] in a medium devoid of $MgCl_2$.

We have thus carried out the glutaraldehyde fixation under both conditions, and found that fixing the chloroplasts with or without $MgCl_2$ had little effect on the ability of glutaraldehyde to protect against alkaline pH. Fixation in the presence of $MgCl_2$ did lead to some inactivation after treatment at pH 9.3, but this was also seen for the control, and was undoubtedly due to some $MgCl_2$ remaining (Table I). It can be seen that glutaraldehyde fixation does not significantly protect against the synergistic effects of alkaline pH and $MgCl_2$, in accordance with the previous results of Briantais et al. [4] for alkaline pH plus light. Thus, it would seem that the sites or modes of action of Tris and alkaline pH are not the same; glutaraldehyde protecting against the effects of the former but not the latter.

At lower pH (7.5) Mg^{2+} did not induce inactivation, but was still able to change the functioning of the water-splitting enzyme as compared with monovalent cations. This was detected via an increase in the damping of the oxygen sequences ($\sigma_{KCl} = 0.4$, $\sigma_{MgCl_2} = 0.7$). In this case also, glutaraldehyde fixation was not able to impede the effect of $MgCl_2$ on the oxygen sequences at pH 7.5 (Table II). It must be noted that the spill-over was minimum with 100 mM KCl or 20 mM $MgCl_2$ and could not be implicated in the modifications described.

These results indicate that if protein structural changes are involved in these phenomena, they can only be of a minor nature. It is more likely that the effects observed are due to ionic interactions of $MgCl_2$ and other cations with the water-splitting enzyme, leading to the release of manganese at pH > 9 as detected by EPR. These cation effects may be correlated with those observed by Åkerlund [22], who has shown that high concentrations of monovalent cations (0.25–0.5 M) added to inside-out thylakoid vesicles inhibit oxygen evolution coinciding with the release of a protein necessary for oxygen evolution.

It is possible that the changes in the damping of the oxygen sequences are due to a loosening of the binding of manganese to the enzyme of water oxidation by the presence of divalent cations. This effect is pH dependent. At pH 6 the damping was low both for KCl ($\sigma = 0.33$) and $MgCl_2$ ($\sigma = 0.30$). This may be because at low pH the binding of $MgCl_2$ is weaker than at high pH [23].

The σ values obtained at pH 7.5 in the presence of $MgCl_2$ are identical to those observed for *Chlorella* cells. If the Mg^{2+} concentration inside *Chlorella* is the same as that observed in intact chloroplasts of peas or spinach, then perhaps the high damping of the O_2 sequences in *Chlorella* is due to the internal pH of dark-adapted *Chlorella* being slightly above pH 7.

A similar supposition was also made by Maison-Peteri [24] based on the absence of double hits in intact *Chlorella* at pH > 7 for chloroplasts and glutaraldehyde-treated *Chlorella,* in contrast to pH 6 where double hits were detected.

In conclusion, Mg^{2+} induces modifications of the functioning of the water-splitting site of Photosystem II at pH > 7, which are probably independent of protein structural changes as they are not suppressed by glutaraldehyde fixation.

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