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Membrane Organization of the Desiccation-Tolerant Moss *Tortula ruralis* in Dehydrated States[1]

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ABSTRACT

Membrane organization of the desiccation tolerant moss *Tortula ruralis* was studied in several intensely dehydrated states (75% relative humidity [RH], 90% RH, plasmolysis in molar salt, freezing to −20°C) by ^{31}P nuclear magnetic resonance and ultrastructural analyses. Both methods revealed that even at 75% RH (−400 bars), the moss cellular membranes retained extended phospholipid bilayers. Ultrastructural analyses of the fully hydrated moss showed an extensive proliferation of membrane vesicles in the endoplasmic reticulum. During dehydration, these vesicles form layers of membrane under the plasmalemma and in some cases appear to fuse with the surface membrane. This suggests that these vesicles may serve as a reservoir of membranes to accommodate for membrane surface area changes during desiccation and subsequent rehydration.

The moss *Tortula ruralis* ([Hedw.] Gaertn., Meyer and Scherb.) can tolerate prolonged conditions of extreme dessication including drying and storage over activated silica gel (15). The removal of water from the cell during dehydration (similarly freezing) results in protoplast shrinkage. Consequently, cellular membranes, especially the plasma membrane, are subjected to mechanical stresses resulting from surface area changes during cell collapse and from disruptions of the water status which is critical for the maintenance of phospholipid lamellar lattice and integrity. In cells which tolerate such stresses, it is understood that whatever form the membranes assume in the desiccated or frozen state, the compartmentation of the cell has to be restored within seconds upon re-expansion during rehydration or thawing. The ability of *T. ruralis* to withstand repeated cycles of hydration—dehydration and extreme freezing makes it an ideal system for study of the nature and organization of membranes in the desiccated and frozen states.

In this study, we report the organization of cellular membranes in *T. ruralis* in the dehydrated condition (desiccated, plasmolyzed, and frozen states) using biophysical (^{31}P NMR[2]) and ultrastructural methods.

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[2] Abbreviation: NMR, nuclear magnetic resonance.

MATERIALS AND METHODS

Green upper gametophytic portions of *T. ruralis* were collected, trimmed, and dried as described by Schonbeck and Bewley (15). The dried moss was stored in jars containing silica gel. Equilibration of the moss to different degrees of hydration was carried out by placement of the dried moss in 7- × 9-cm sealed glass jars for 24 to 48 h at room temperature over 50-ml mixtures of anhydrous glycerine and water adjusted to give a RH of either 90% or 75% (9). Complete rehydration was done by placing the dried moss on moist filter paper. Plasmolysis of rehydrated moss was carried out by immersing 2-mm distal leaf portions of the moss in a 4 M balanced salt solution (NaCl:$CaCl_2$, 9:1) for 15 min.

^{31}P NMR experiments were carried out at 25°C in a Bruker 250 NMR spectrometer equipped with a 10-mm broadband probe operating at 101.3 MHz. Moss samples (equivalent to 0.25 g dry wt) equilibrated for 24 h to different degrees of hydration (fully hydrated, 90% RH, 75% RH, plasmolyzed and dry) were packed into 10-mm outer diameter quartz tubes to give a full volume of 2 ml. In the case of 90% RH or 75% RH equilibrated moss, open-ended glass tubes containing glycerol-water mixtures to give the appropriate RH were also placed inside the sealed NMR tubes in order to maintain the same RH for the duration of the run.

Spectra were acquired with a normal spin echo pulse sequence (13) (90°-τ-180°C-τ-Acquisition) in which the 90° pulse was 14.5 μs and echo delays were 70 μs. Maximum decoupling conditions (2.2 G) were used during pulsing and acquisition, and the decoupler was gated to prevent sample heating. Free Induction Decays of 2K data points and 100 kHz sweep-width were transformed with exponential enhancement and 2K zero fill.

Lipid Extraction. One g (dry weight) of moss was ground to fine pieces in a Wiley Mill and then extracted according to de la Roche *et al.* (5). The phospholipids were separated from other lipids by chromatography on a silica-acid column (14). Carotenoid and Chl was removed by elution with chloroform followed by acetone. Phospholipids were eluted by methanol. The eluted phospholipids were further purified on preparative TLC plates according to the method of Gardner (6). Multilayered liposomes were prepared by shaking the dried phospholipid in 0.1 M KCl. Lipid phosphorus was determined by the method of Allen (1).

Fixation in the Desiccated State. Osmium tetroxide vapor fixation of the moss previously equilibrated to 90% or 75% RH were carried out in sealed glass jars under the specified RH. Although the moss samples turned black in less than 24 h, the fixation procedure was continued for 1 week. The fixed moss did not expand when rehydrated and subsequent fixation in buffered 2% osmic acid did not result in further ultrastructural alterations. Moss samples dried over silica gel and subsequently placed in

jars with the RH equivalent to the laboratory (35–45% RH) were not able to be fixed by osmic acid even after 2 weeks.

Fixation during Plasmolysis. Moss distal leaf portions initially plasmolyzed for 15 min in a 4 M salt-balanced solution were fixed in the same solution containing 2% osmic acid overnight at 2°C.

Fixation in the Frozen State at −20°C. Moss distal leaf portions were frozen to −20°C at −5°C/h. The leaves were then fixed in osmic acid in the frozen state at −20°C using the method of McKenzie *et al.* (8) modified by Singh (16).

All fixed samples were dehydrated in a graded series of 25%, 50%, 75%, 90% and absolute ethanol with 5-min intervals between grades. The dehydrated samples were then infiltrated for 30 min with equal volumes of ethanol: LR White resin (Polaron Equipment Ltd., Watford, England) followed by infiltration with 100% LR white resin for 72 h with frequent changes of fresh resin. Polymerization was carried out overnight in closed gelatin capsules in an oven set at 60 ± 0.2°C. Thin sections were stained with aqueous uranyl acetate followed by lead citrate and viewed on a Philips 300 electron microscope.

RESULTS

^{31}P NMR spectra of fully hydrated leaves and of isolated total phospholipids of *T. ruralis* are presented in Figure 1. The spectrum of 0.25 g dry weight of fully hydrated moss (Fig. 1A) consists of a broad central line superimposed on an anisotropic powder pattern. The central resonance at 0 ppm is diagnostic of phosphorus-containing compounds undergoing isotropic motion and in this case represents Pi. The resonance is broadened due to the viscous nature of the cellular cytoplasm and the presence of intracellular ions. The anisotropic pattern of the spectrum is characteristic of lipid phosphorus in extended bilayers and appears to be composed of two superimposed powder patterns in which the chemical shift anisotropies ($\Delta\sigma_{CSA}$) are approximately

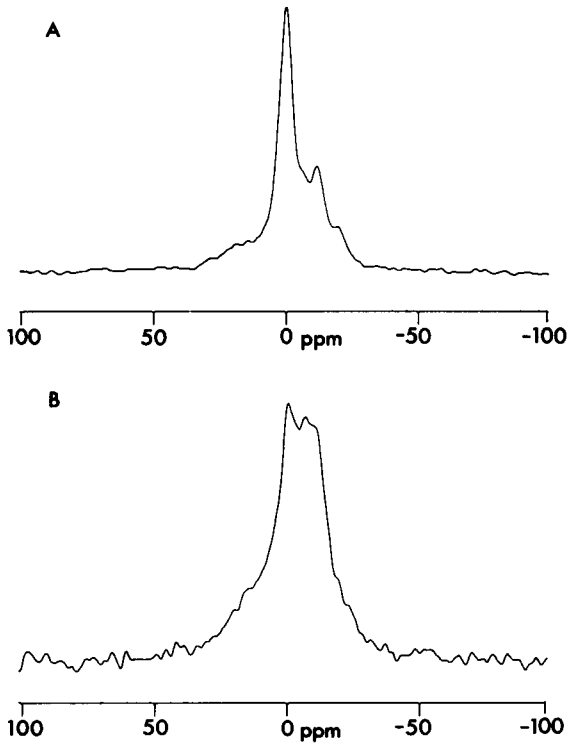

FIG. 1. ^{31}P NMR spectra of fully hydrated *T. ruralis* and total phospholipid isolated from moss. A, Fully hydrated moss (0.25 g dry weight); B, isolated phospholipid (from 1 g dry weight of moss).

35 and 50 ppm. This behavior is not a spectral artifact but was consistently observed with fully hydrated moss, and may suggest a segregation of phospholipid domains of different residual anisotropy (2). Liposomes made from isolated total phospholipids of *T. ruralis* (14 mg from 1 g dry weight of moss) gave a ^{31}P NMR spectrum (Fig. 1B) in which the anisotropic component has similar overall $\Delta\sigma_{CSA}$ to that in Figure 1A. However, the segregation of powder patterns was not as distinct possibly because the liposomes were a mixture of all phospholipid species. The presence of an isotropic component at 0 ppm is likely due to the presence of very small liposomes and single bilayer vesicles which were formed in addition to large liposomes, and is generally observed in liposome spectra of natural phospholipid mixtures. Distribution in liposome sizes could be observed under the light microscope.

The ultrastructure of the fully hydrated moss is presented in Figure 2A. A proliferation of vesicles could be discerned throughout the cytoplasm. Some of these vesicles could be seen in various stages of fusion with the plasmalemma. The presence of numerous vesicles and electron dense bodies is characteristic of these cells and has been reported previously (20).

Figure 3 shows ^{31}P NMR spectra of moss leaves at different levels of hydration. The quantity of water in 90% RH (−140 bars) and 75% RH (−400 bars) equilibrated moss are 30% and 18% of the dry weight, respectively (15). As the extent of hydration decreases, the spectrum becomes broader and the total signal decreases. However, even at 75% RH (Fig. 3B), the anisotropic component is still evident and suggests that bilayer formation is retained. When the moss was equilibrated to 30 to 40% RH or dried over silica-gel, a ^{31}P NMR 'rigid limit' spectrum (Fig. 3C) typical of immobilized phospholipids was observed (3). Detailed analysis of this spectrum was not possible because of poor signal-to-noise ratio but the fact that bilayers are still present cannot be precluded. Rehydration of the dried moss restored the spectrum to that observed in Figures 1A or 3A, indicating there was no permanent disruption of the bilayer. It was observed that when the moss died under hydrated conditions due to overheating, the spectrum showed only a broad isotropic component.

The ultrastructure of cellular membranes of moss leaves at 75% RH is shown in Figure 2B. Membranes of 90% RH fixed moss were ultrastructurally similar. The protoplasts of the osmium vapor-fixed cells were contracted and stained intensely. Nevertheless, even at 75% RH, trilamellar characteristics of the plasmalemma, cytoplasmic vesicles, and densely compressed chloroplast grana (not shown) could be discerned. The cytoplasmic vesicles were frequently compressed and could either be seen as stacks of extended bilayers under the plasmalemma or as vertical finger-like invaginations (Fig. 2B, inset) from the plasmalemma into the cytoplasm. Whether these invaginations were a result of folding of the plasmalemma or a result of fusion with the vesicles is not known. Electron dense bodies observed in hydrated cells were still present in the partially desiccated state (not shown). It was not possible to fix moss cells which were either equilibrated on the bench (35–45% RH) or dried over silica-gel. Fixation of dried moss in nonaqueous solvents containing osmic acid was also unsuccessful. The low sensitivity of ^{31}P NMR necessitated a long accumulation time for good signal-to-noise ratios in obtaining the lipid phosphorus component and precluded the possibility of using a Dante pulse sequence (4) to allow cancellation of the Pi peak on the same sample following the normal pulse sequence data acquisition. The ^{31}P NMR spectrum of moss leaves plasmolyzed in 4 M balanced salt (Fig. 4) is similar to that in the fully hydrated state suggesting that in the plasmolyzed state the structural organization of lipid phosphorus was not altered. This was confirmed by osmic acid fixation of moss cells in 4 M salt (Fig. 2C) showing that the plasma membrane trilamellar ultrastructure was retained. Figure

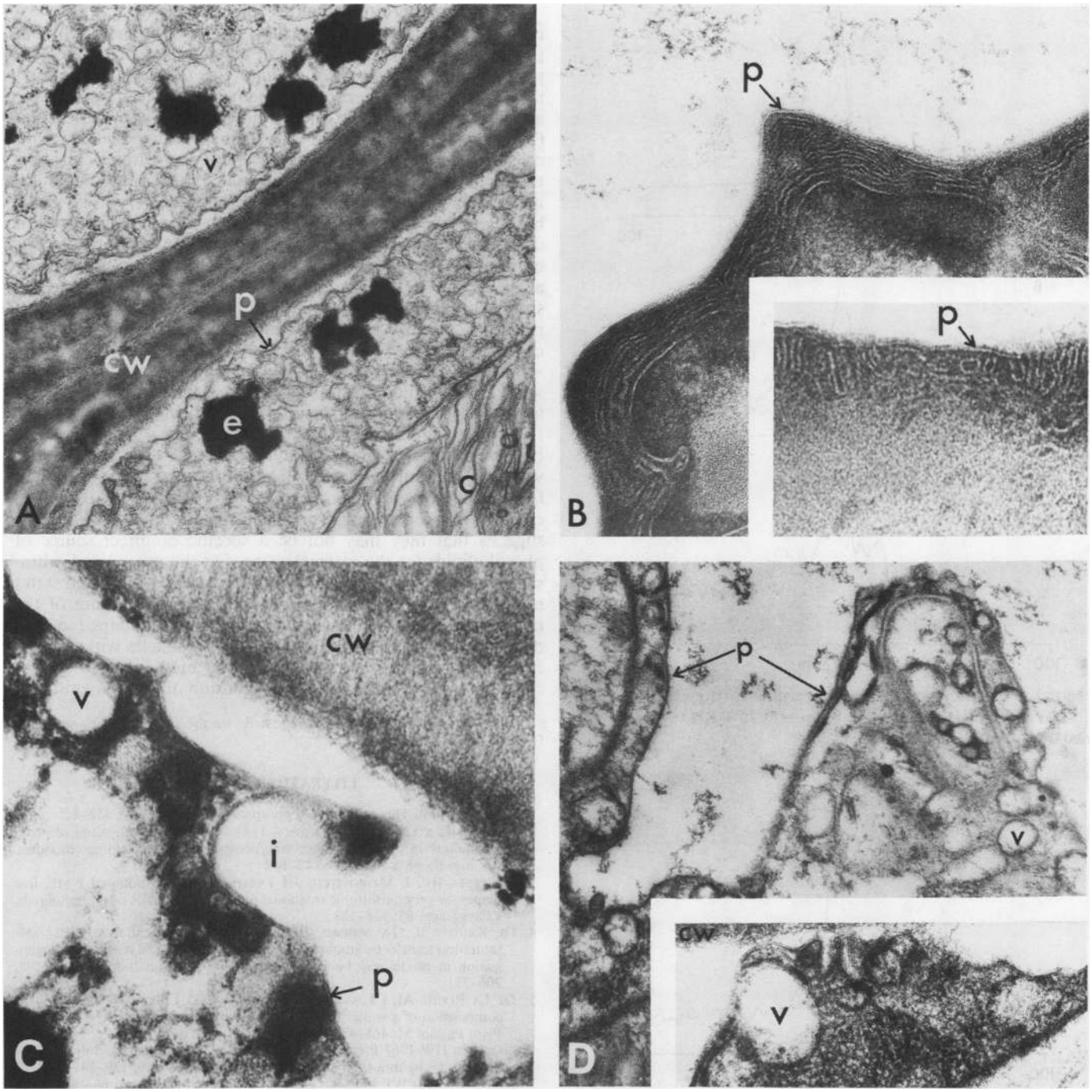

FIG. 2. Ultrastructure of *T. ruralis* cells under different degrees of dehydration. A, Fully hydrated state (× 32, 091); B, equilibrated to 75% RH (× 120,360; inset, × 218,340); C, plasmolyzed in 4 M balanced salt (× 138,140); D, fixed in the frozen state at −20°C (×63,094; inset, × 84,290). Abbreviations: c, chloroplast; cw, cell wall; e, electron dense bodies, i, invagination; p, plasma membrane; v, vesicles.

2C also suggests that some invaginations (i) formed during retreat of the plasmalemma from the wall during plasmolysis may result from fusion of some of the cytoplasmic vesicles with the surface membrane. The outcome is extra membrane material for concave-types of membrane expansion during plasmolyses.

^{31}P NMR spectra of hydrated moss leaves during freezing to low temperatures (−20°C) were relatively uninformative in that a broad rigid-limit low signal-to-noise spectrum similar to Figure 3C was obtained. Motional restriction of the lipid phosphorus during dehydration was probably exacerbated by low temperatures resulting in a spectral shape similar to that obtained for silica-gel dried moss (Fig. 3C). However, fixation of moss cells during freezing at −20°C revealed that the cellular membranes retained their phospholipid lamellar lattice (Fig. 2D). Again, cytoplasmic vesicles could be seen just beneath and in some instances in close contact with the surface membrane.

CONCLUSIONS

Combined ^{31}P NMR and ultrastructural analyses of *T. ruralis* in dehydrated states (desiccated, plasmolyzed, and frozen) suggested that even at very low water content (75% RH equilibrated, −400 bars) their membranes retained bilayers. Similar ^{31}P NMR observations have been made in *Typha* pollen by Priestley and

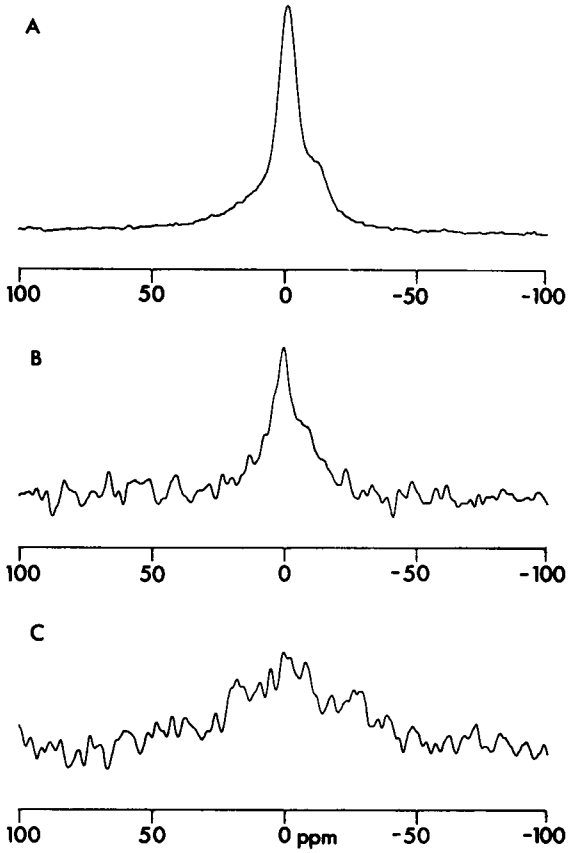

FIG. 3. ^{31}P NMR spectra of *T. ruralis* at different degrees of hydration. A, Moss at 90% RH (−140 bars); B, moss at 75% RH (−400 bars); C, dried over silica gel (about −6000 bars).

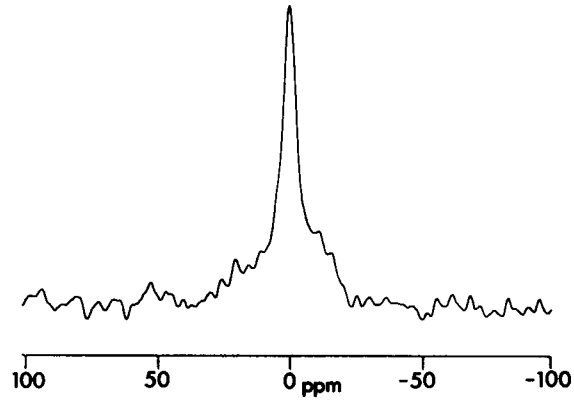

FIG. 4. ^{31}P NMR spectrum of *T. ruralis* plasmolyzed in 4 M balanced salt.

de Kruijff (12) and it has also been shown ultrastructurally that one mechanism of membrane injury in nonhardy rye cells during dehydrative extracellular freezing or plasmolysis is the loss of crystalline phospholipid bilayers (17). Although we were not able to evaluate membrane organization in the very dry state (0.1 g H_2O/g dry weight) because of low sensitivity, we feel that moss leaves equilibrated at 75% RH (−400 bars) already represented a state of desiccation or equivalent freeze-induced dehydration that only very few plants can survive. Taken together, these results suggest that retention of phospholipid lamellar lattice during dehydration is a prerequisite for successful compartmentation upon rehydration and thus tolerance to extreme desiccation.

One interesting ultrastructural observation in *T. ruralis* is the proliferation of numerous vesicles in the cytoplasm. Similar proliferations have been observed in cold-hardened secondary phloem parenchyma cells of *Robinia pseudoacacia* (11), in cold-hardened mulberry cortical parenchyma cells (10), and in rye and wheat epicotyl cells hardened either by cold or by desiccation (Singh and Cloutier, unpublished). In all cases studied, plant cells capable of cold hardening develop these features concomitant with the development of tolerance to dehydration or freezing. However, the manner in which this proliferation of membrane vesicles can alleviate dehydration damage has not been described. The presence in dehydration-tolerant cells of reservoirs of nonbilayered membrane material deleted and then made available for isolated protoplast contraction and expansion, respectively, during dehydration-hydration cycles has been proposed (7, 21, 22). However, ultrastructural analyses of whole cold-hardened rye cells fixed in the frozen state at −10 and −20°C suggested that the surface membrane can either be conserved by invaginations during freezing in tolerant cells (18) or in fact be subjected to expansion stresses during plasmolysis (19). In the moss, numerous electron dense bodies were observed to be present in the plasmolyzed and desiccated states. These bodies were also present in the fully hydrated state (20) and, in cells fixed during rehydration, no bilayered membraneous structures could be seen emanating from them (Singh, unpublished). This suggests that they may not be a specific or direct source of phospholipids for the plasmalemma. On the other hand, ultrastructural observations of the moss in various dehydrated states reported in this study suggest that the lamellar nature of the plasmalemma and cytoplasmic vesicles were retained during dehydration. Furthermore, fusion of these vesicles with the surface membrane during cell dehydration may supply the moss cell with ample membrane for reexpansion during rehydration.

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