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LOCALIZATION OF THE AMINO PHOSPHOLIPIDS IN SARCOPLASMIC RETICULUM MEMBRANES REVEALED BY TRINITROBENZENE-SULFONATE AND FLUORODINITROBENZENE

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Summary

The chemical probes for amino compounds 2,4,6-trinitrobenzenesulfonate (TNBS) and 1-fluoro-2,4-dinitrobenzene (FDNB) were utilized to determine the localization of the amino phospholipids in the sarcoplasmic reticulum membranes. At low concentrations (<1 mM), TNBS does not penetrate the sarcoplasmic reticulum membrane, while FDNB readily penetrates it. The results show that about 70% of the total phosphatidylethanolamine is located on the external surface of the membrane, about 20% is on the internal surface and 10% is probably strongly interacting with the proteins since it is not accessible to the probes. In contrast, most of the phosphatidylserine is located on the inner surface of the membrane. This molecular distribution of the amino phospholipids supports a structural assymmetry of the sarcoplasmic reticulum membrane.

Introduction

The chemical probes for amino compounds, trinitrobenzenesulfonate, fluorodinitrobenzene and others, have been employed to demonstrate the asymmetric distribution of the amino phospholipids in erythrocyte membranes [1—4], which was confirmed by use of phospholipases [5,6]. Phospholipid asymmetry also has been reported in membranes of endoplasmic reticulum [7] and in artificial membranes [8,9]. Likewise, studies performed with sarcoplasmic reticulum membranes [10—17] show that the main proteins (Ca^{2+}-ATPase and Ca^{2+}-precipitating protein), as well as the amino lipids, are asymmetrically distributed within the membrane. Using the fluorescent probe, Fluram, Hasselbach and Migala [16] found that most of the total amino phos-

Abbreviations: TNBS, 2,4,6-trinitrobenzenesulfonate; FDNB, 1-fluoro-2,4-dinitrobenzene.

pholipids are localized on the external leaflet of the membrane. However, the localization of the different amino lipids species (phosphatidylethanolamine and phosphatidylserine) was not differentiated.

In this work, the non-penetrating probe TNBS and the penetrating probe FDNB were utilized to investigate the molecular arrangement of the amino phospholipids in the sarcoplasmic reticulum membrane.

Materials and Methods

Isolation of sarcoplasmic reticulum

Sarcoplasmic reticulum vesicles were isolated from rabbit white skeletal muscle as previously described [18]. The protein was measured by the biuret method [19] using bovine serum albumin as a standard.

Labelling of the sarcoplasmic reticulum membranes with TNBS or FDNB

Sarcoplasmic reticulum membranes (20 mg) were incubated for 3 h at 23°C in a medium containing 50 mM KCl, 0.1% albumin, 100 mM $NaHCO_3$, 0.25 M sucrose, several concentrations of TNBS or FDNB and 0.5% of sodium deoxycholate (if present) in a total volume of 20 ml at pH 8.2. After the incubation period, the suspensions of the membranes not solubilized were centrifuged 20 min at $40000 \times g$. The supernatants were discarded and the pellets washed twice with 20 ml of the suspending medium, without the probes.

The solubilization of the sarcoplasmic reticulum membrane by butanol was carried out as described by Maddy [20], and the extracted lipids were directly incubated in the medium described above.

Phospholipid analysis

The membrane lipids were extracted in $CHCl_3/CH_3OH$ (1 : 1, v/v) mixtures [21]. The extracts were centrifuged and the lipid fraction was evaporated in a rotatory evaporator. The final lipid residues were dissolved in $CHCL_3/CH_3OH$ (2 : 1, v/v) mixtures for spotting on thin layer chromatography plates (silica Gel G, type 60, Merck). The phospholipids were separated by uni-dimensional chromatography in $CHCl_3/CH_3OH/NH_4OH/H_2O$ (70 : 30 : 4 : 1, v/v), or by two-dimensional chromatography using this solvent in the first dimension and $CH_3/CH_3OH/CH_3COOH/H_2O$ (90 : 40 : 12 : 2, v/v) in the second one. The lipids which reacted with TNBS or FDNB were identified by their yellow colour, and the free lipids were located using iodine vapors. The quantitative analysis was determined by measuring the amount of P_i by the method of Bartlett [22] in the scraped spots previously digested in 70% $HClO_4$ at 190°C [23].

Electron microscopy

At the end of the labelling period, samples of control and TNBS or FDNB treated vesicles were diluted to give a final concentration of 0.1 mg of sarcoplasmic reticulum protein per ml. Droplets of these suspensions (3 μl) were negatively stained for 10 min with 3 μl of 2% phosphotungstic acid (pH 7.2) on formvar-coated grids (400-mesh). After drying, they were observed in a Siemens Elmiskop 101 electron microscope at 80 kV accelerating voltage and 10 μA emission current. Pictures were taken at a magnification of 20000.

Reagents

All reagents were analytical grade.

Results

Labelling of the amino phospholipids by TNBS in sarcoplasmic reticulum membranes

The probe 2,4,6-trinitrobenzenesulfonate (TNBS) has been revealed as a good tool to show the asymmetry of the erythrocyte membrane [1,3]. In this work, TNBS and FDNB were utilized to determine the localization of the amino phospholipids in membranes of sarcoplasmic reticulum which contain approx. 16% of phosphatidylethanolamine and approx. 1.5% of phosphatidylserine relative to the total amount of phspholipid (Fig. 1).

Fig. 1 shows that as the concentration of TNBS increases, initially the amount of phosphatidylethanolamine labelled by TNBS in intact sarcoplasmic reticulum vesicles also increases until it reaches a plateau at approx. 0.6—1.0 mM TNBS, for which about 70% of the total phosphatidylethanolamine is reacted with the probe. Finally, at higher TNBS concentrations, the labelling is increased again (about 90% of the total phosphatidylethanolamine being labelled at 2.0 mM TNBS). If the reaction with the probe takes place after solubilization of the membranes with sodium deoxycholate or butanol, the maximal amount of phosphatidylethanolamine labelled (about 90%) is reached at approx. 0.6 mM of TNBS. These results indicate that in intact vesicles the high concentrations of TNBS (>1 mM) facilitate the penetration of the probe probably because the sarcoplasmic reticulum membrane is disrupted and the internal phosphatidylethanolamine molecules are exposed to TNBS. In contrast

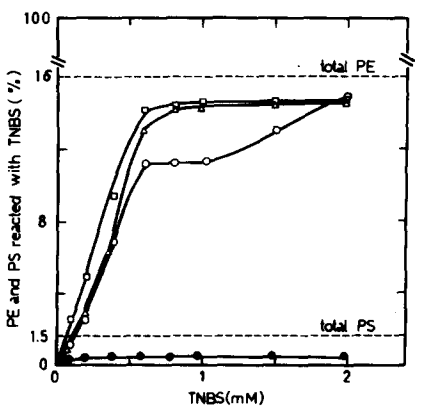

Fig. 1. Labelling of the amino phospholipids by TNBS in membranes of sarcoplasmic reticulum. The sarcoplasmic reticulum vesicles were incubated in the presence of 0.0, 0.1, 0.2, 0.4, 0.6, 0.8, 1.0, 1.5 and 2.0 mM of TNBS as described in the text. The phospholipids were separated by thin layer chromatography and quantified by measuring the P_i in the scraped spots. ○———○, Amount of phosphatidylethanolamine reacted with TNBS in intact membranes. △———△, Amount of phosphatidylethanolamine reacted with TNBS in membranes solubilized by sodium deoxycholate. □———□, Amount of phosphatidylethanolamine reacted with TNBS after direct incubation of the lipids obtained by solubilization of the membranes with butanol. ●———●, Amount of phosphatidylserine reacted with TNBS in intact membranes. PE, phosphatidylethanolamine; PS, phosphatidylserine.

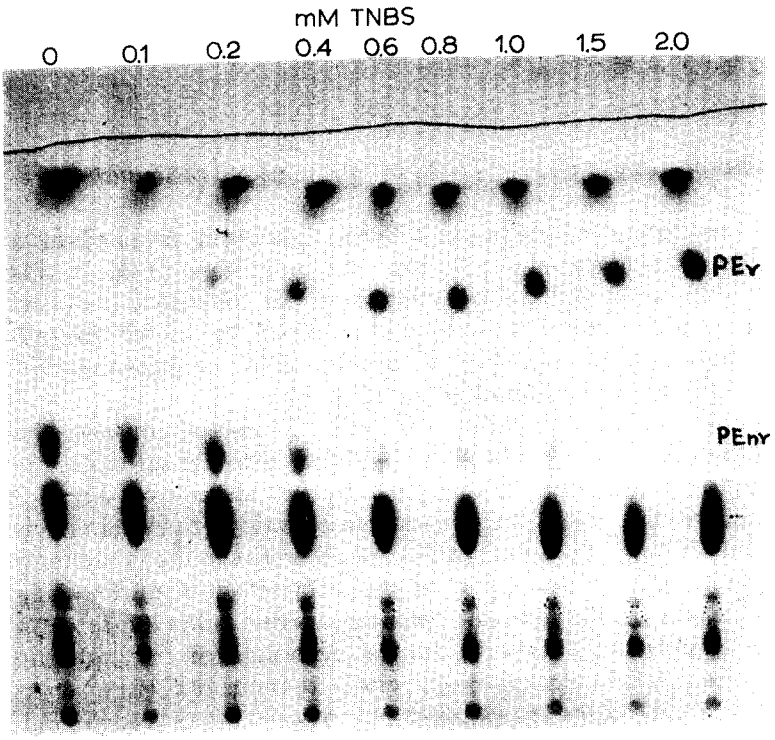

Fig. 2. Unidimensional chromatography for phospholipid separation after reaction of sarcoplasmic reticulum with various concentrations of TNBS. The reaction was carried out as described in Fig. 1. The chromatography plates were developed in $CHCl_3/CH_3OH/NH_4OH/H_2O$ (70 : 30 : 4 : 1, v/v). PE_r, phosphatidylethanolamine reacted with TNBS. PE_{nr}, phosphatidylethanolamine not reacted with TNBS. The concentrations of TNBS (mM) are indicated on the top of the picture.

with the phosphatidylethanolamine, only a negligible fraction of the phosphatidylserine appears labelled by TNBS in the intact membranes, indicating that the phosphatidylserine is not easily accessible to the probe.

Fig. 2 shows the phospholipid separation by unidimensional chromatography after reaction of the membranes with several concentrations of TNBS. Since the reaction with TNBS alters the chromatographic mobility and the colour of the phosphatidylethanolamine observed on the plates, it is possible to differentiate between the free and the reacted phosphatidylethanolamine. Thus, we can observe that as the concentration of TNBS is increased there is progressive enhancement in the spots of reacted phosphatidylethanolamine, which corresponds to the disappearance of those phosphatidylethanolamine spots not reacted with TNBS. The lysophosphatidylcholine, phosphatidylinositol, sphingomyelin, phosphatidylcholine and the neutral lipids are not altered by the probe, which reacts specifically with the amino phospholipids (phosphatidylethanolamine and phosphatidylserine).

Fig. 3 shows the phospholipid separation by two-dimensional chromatography which permits to obtain a good separation of the free and reacted phosphatidylserine from the other phospholipids (sphingomyelin, phosphatidylinositol and lysophosphatidylcholine).

Fig. 3. Two-dimensional chromatography for phospholipids separation after reaction of sarcoplasmic reticulum with 1 mM TNBS. The sarcoplasmic reticulum vesicles were incubated with 1 mM TNBS as described in the text. The chromatography plates were developed in $CHCl_3/CH_3OH/NH_4OH/H_2O$ (1st solvent) and $CHCl_3/CH_3OH/CH_3COOH/H_2O$ (2nd solvent). Lys-PC, lysophosphatidylcholine; Sph, sphingomyelin; PS_{nr}, phosphatidylserine not reacted with TNBS; PI_n, phosphatidylinositol; PS_r, phosphatidylserine reacted with TNBS; PC, phosphatidylcholine; PE_{nr}, phosphatidylethanolamine not reacted with TNBS; PE_r, phosphatidylethanolamine reacted with TNBS; NL, neutral lipids.

Since the biochemical analysis (Fig. 1) indicates that high concentrations of TNBS probably disrupt the membrane, exposing the internal phosphatidylethanolamine molecules to the probe, it is of interest to observe by electron microscopy the nature of the structural aspect of the sarcoplasmic reticulum vesicles treated with several concentrations of TNBS.

Fig. 4a shows sarcoplasmic reticulum vesicles treated with 0.2 mM of TNBS. Closed vesicles of various shape and size can be observed. If the TNBS concentration increases (1 mM), the vesicles appear more heterogenous in shape and size (Fig. 4b) and at the highest concentration of TNBS (2 mM), deformed vesicles mixed with broken fragments are predominant (Fig. 4c).

Labelling of the amino phospholipids by FDNB in sarcoplasmic reticulum membranes

The probe 1-fluoro-2,4-dinitrobenzene (FDNB), which also reacts with the lipid amino groups [24] and has been described as a penetrating probe in erythrocyte membranes [2,25], was utilized in this work to label the amino phospholipids in membranes of sarcoplasmic reticulum.

Fig. 5 shows that the amount of phosphatidylethanolamine reacted with FDNB enhances as the concentration of the probe is increased reaching a maxi-

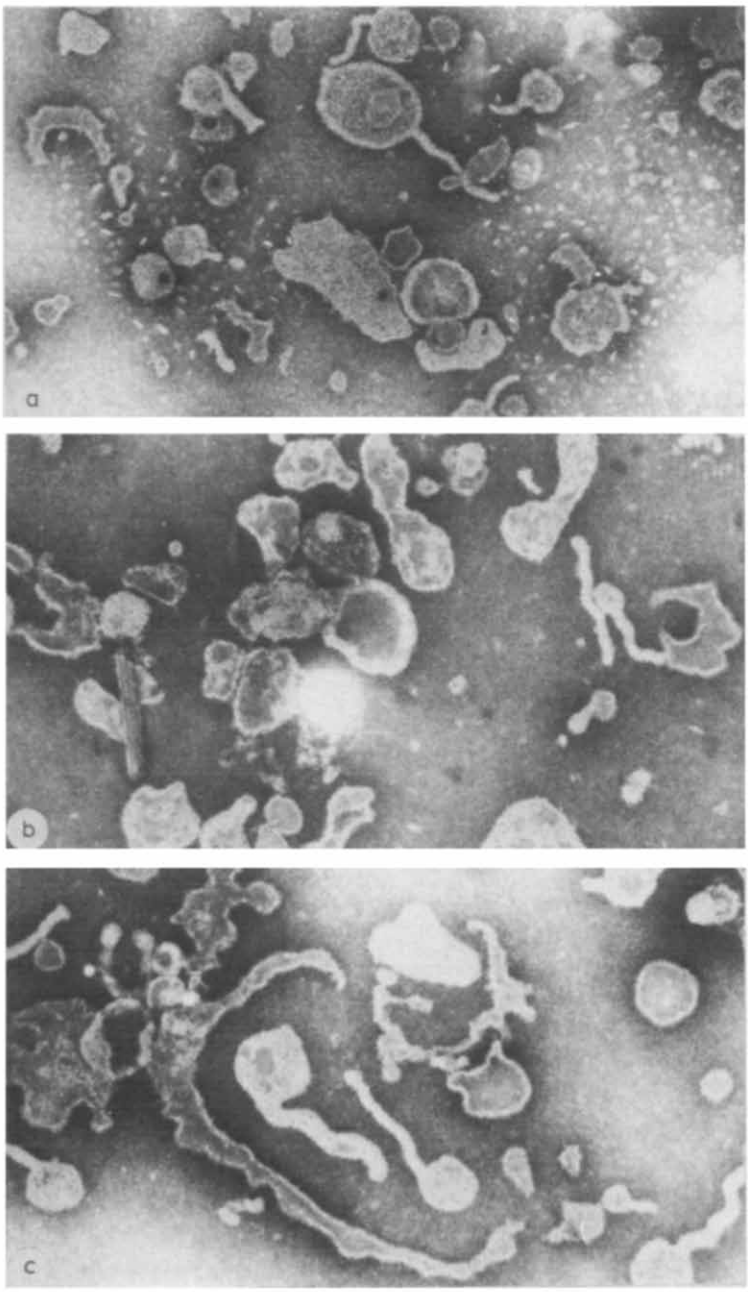

Fig. 4. Electron micrographs of sarcoplasmic reticulum vesicles treated with various concentrations of TNBS. Negative staining with 2% phosphotungstic acid (×56000). a, Treatment with 0.2 mM TNBS: closed vesicles of various size can be observed. b, Treatment with 1 mM TNBS: the vesicles appear more heterogenous, with protuberances. c, Treatment with 2 mM of TNBS: The vesicles are deformed and broken fragments can be seen.

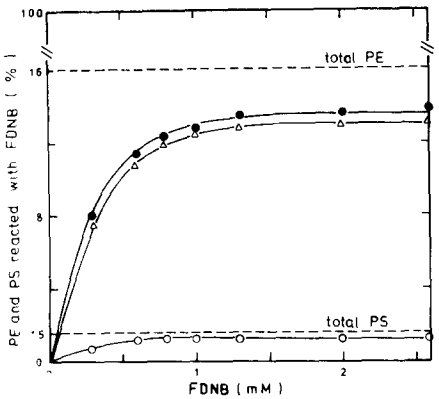

Fig. 5. Labelling of the amino phospholipids by FDNB in membranes of sarcoplasmic reticulum. The sarcoplasmic reticulum vesicles were incubated in the presence of 0.3, 0.6, 0.8, 1.0, 1.3, 2.0 and 2.6 mM of FDNB as described in the text and the phospholipids were analysed as in the experiment of Fig. 1. △———△, Amount of phosphatidylethanolamine reacted with FDNB in intact membranes. ●———● Amount of phosphatidylethanolamine (PE) reacted with FDNB in membranes solubilized with sodium deoxycholate. ○———○, Amount of phosphatidylserine (PS) reacted with FDNB in intact membranes.

mal amount (about 80% of the total phosphatidylethanolamine) at 1 mM of FDNB. In contrast with TNBS, above this concentration, the extent of labelling remains constant, which indicates that all phosphatidylethanolamine available to FDNB is saturated at the concentration of 1 mM of FDNB. Furthermore, the intact sarcoplasmic reticulum and that solubilized with sodium deoxycholate showed a similar extent of reaction between the amino phospholipids and the FDNB (about 85% of the total phosphatidylethanolamine was labelled above 1 mM FDNB concentration). On the other hand, while TNBS did not

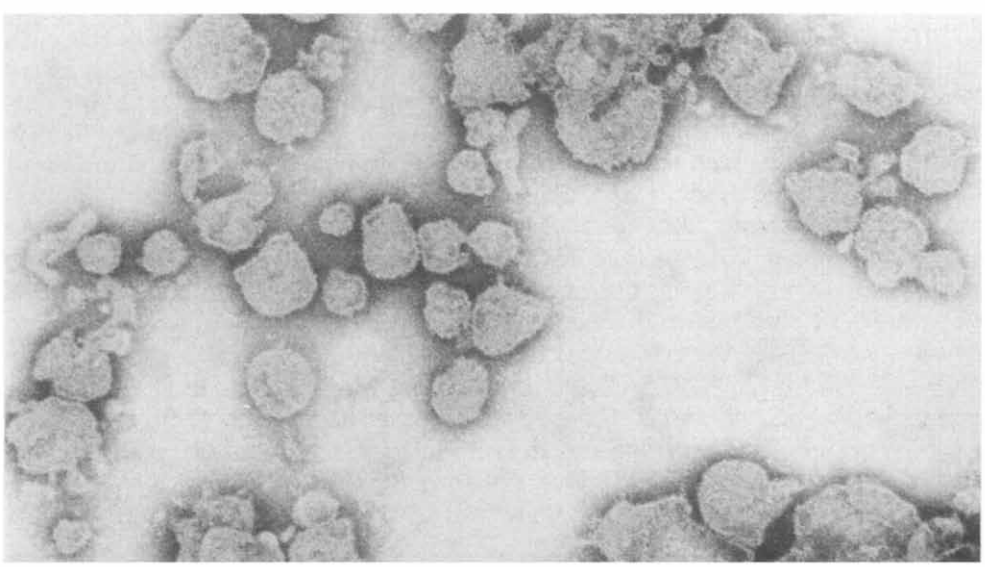

Fig. 6. Electron micrograph of sarcoplasmic reticulum vesicles treated with 2 mM FDNB. Negative staining with 2% phosphotungstic acid (X74400). The vesicles appear more homogenous than those treated with TNBS.

react extensively with phosphatidylserine (Fig. 1), it was significantly labelled by FDNB (Fig. 5).

The electron micrograph of FDNB treated sarcoplasmic reticulum (Fig. 6) shows that the vesicles appear more homogenous than those treated with TNBS, indicating that the FDNB molecule is small enough to penetrate the membrane without damaging it.

These results indicate that while FDNB easily penetrates the sarcoplasmic reticulum membrane, only high concentrations of TNBS permit its penetration due to disruption of the membrane. On this basis, the use of these chemical probes allowed us to determine that while most of the phosphatidylserine is internally localized on the sarcoplasmic reticulum membrane, about 70% of the total phosphatidylethanolamine is located on the external surface, 20% is on the internal surface and the other 10% is not accessible to the probe, probably due to a strong interaction with the protein components of the membrane.

Discussion

To investigate the molecular distribution of the membrane components utilizing chemical probes, it is necessary to study the conditions of their penetration through the membrane. The probe TNBS, which reacts with the amino compounds, has been described as a non-penetrating probe in membranes of erythrocytes [1,3]. In this work, the results show that under certain conditions, the sarcoplasmic reticulum membrane is not penetrated by TNBS. After reaching a level of saturation at which 70% of the total phosphatidylethanolamine is labelled by the probe (Fig. 1), increasing the TNBS concentration in the medium above 1 mM permits the labelling of other fraction of the phosphatidylethanolamine (20%). This observation indicates that at high concentrations of the probe (>1 mM), the vesicular membranes are destroyed so that the internal surface is exposed to TNBS. This interpretation is supported by the electron micrographs, which show that above the critical level (1 mM) the sarcoplasmic reticulum vesicles appear altered in shape and size (Fig. 4a, b, c). Furthermore, Fig. 1 shows that the solubilization of the membranes by deoxycholate or butanol becomes accessible at 0.6 mM of TNBS, the fraction of phosphatidylethanolamine (approx. 20%) which, in intact membranes, is available to the probe only above 1 mM. In contrast with the TNBS reaction, intact and solubilized membranes show a similar labelling of the phsophatidylethanolamine by FDNB at the various concentrations used (Fig. 5), indicating a free penetration of this probe through the sarcoplasmic reticulum membrane. In previous works we observed that TNBS [26] and FDNB (Vale, M.G.P., unpublished) inhibit the Ca^{2+}-pump activity of the sarcoplasmic reticulum. However, while the action of TNBS was dependent on the period of preincubation of the membranes with the probe, the inhibitory effect of FDNB was independent of time, which indicates that the sarcoplasmic reticulum membrane is indeed readily penetrated by FDNB and not by TNBS (ref. 26 and unpublished results).

The results reported here show that about 70% of the total phosphatidylethanolamine is located on the external surface of the sarcoplasmic reticulum membrane, about 20% is located on the internal surface and about 10% is prob-

ably tightly bound to the proteins since it is not accessible to the probes. An intimate association between phosphatidylethanolamine molecules and the ATPase enzyme was suggested by Madeira et al. [12]. Moreover, Racker and Eytan demonstrated that phosphatidylethanolamine lipids are required to achieve functional reconstitution of the Ca^{2+}-pump vesicles [27]. Furthermore, in this work it was found that most of the phosphatidylserine is located in the internal leaflet of the lipid bilayer, since it is labelled essentially by FDNB, while TNBS reacts only with a very small fraction. This finding accords with the observation that in intact sarcoplasmic reticulum membranes, the phosphatidylserine is not available to the phospholipase-A enzyme, while it digests promptly the phosphatidylserine in sonicated membranes [12].

These results demonstrate a highly asymmetric arrangement of the amino phospholipids in the sarcoplasmic reticulum membrane where they predominate on the outer surface (approx. 70%). This finding agrees with previous observations obtained by Hasselbach et al. [15,16] using the fluorescent probe Fluram.

On the basis of these results and considering that the outer leaflet is mostly composed of the ATPase enzyme while the inner leaflet is prevalently a lipid monolayer [17], it is plausible to assume that the phosphatidylcholine (which is the most abundant phospholipid of the sarcoplasmic reticulum membrane) is internally localized. Further experiments are being elaborated in this laboratory to show the localization of the phospholipid species which do not contain amino groups.

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