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SPECTROSCOPIC STUDIES OF RAT MAST CELLS, MOUSE MASTOCYTOMA CELLS, AND COMPOUND 48/80—I.

A SPIN-LABEL STUDY OF MEMBRANE FLUIDITIES AND THE EFFECT OF 48/80

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Abstract—Purified rat mast cells, mixed peritoneal leukocytes, and mouse mastocytoma cells take up spin-labeled membrane probes (5-doxyl, 12-doxyl, and 16-doxyl stearic acids and their methyl stearate counterparts) in a manner similar to that of other cell types. The electron spin resonance spectra of these probes suggest that the membrane fluidities in the regions probed are similar in both mast cells and other normal leukocytes of the rat peritoneal cavity. However, the membranes of the mastocytoma cells were significantly more fluid in all of the regions probed. Membrane fluidity differences reported by the methyl stearate probes were greater than those reported by the corresponding fatty acid probes. This suggests that the chemical nature of the probe may contribute significantly to the apparent reported fluidity. The histamine liberator, compound 48/80, did not affect the spectra of any of the probes at concentrations that were magnetically dilute; however, it reduced the degree of spin exchange at high probe concentrations in mastocytoma cells. This suggests that 48/80 may increase the volume of the mastocytoma cell membranes, resulting in reduced intermolecular interactions among the probe molecules.

The rat peritoneal mast cell has been widely studied because of its role in the anaphylactic response [1] and because it is also a useful model system for examining the secretory process [2]. Stimulation of mast cells either by immunological reactions or by chemical stimulators causes changes in the molecular properties of the plasma membrane that result in its sudden fusion with perigranular membranes [3]. The perigranular membranes are also changed and begin to fuse with each other, forming deep channels that communicate with the external environment. During these processes many potent pharmacological agents (including histamine) are released from the exposed granules. The duration of these events is usually less than 10 sec when chemical stimulators are used. Compound 48/80 is a potent chemical stimulator of histamine secretion which probably acts via a pathway separate from that of the immunological response [4]. The final reaction, however, is energy-requiring and noncytotoxic [5].

We have studied the mast cell membrane using spin-labeled fatty acids and esters containing the nitroxide moiety. Because the e.s.r.† spectrum of a nitroxide spin label reflects its rotational motion, the constraint upon that motion produced by the immediate environment is readily discernible. Three of the probes used were stearic acid analogs containing a nitroxide reporter group attached covalently to the 5th (5-DSA), 12th (12-DSA) and 16th (16-DSA) carbon atoms, whereas the remaining three were the analogous methyl stearate derivatives. When incorporated into membranes, each of these nitroxides exhibits an e.s.r. spectrum which is determined by the position of the nitroxide in the probe, as well as by the nature of the local membrane environment. With the aid of these probes, we have examined the membranes of mast cells and of peritoneal and pleural leukocytes and have found differences in: (a) the binding of the stearic acid and methyl stearate probes, (b) the molecular motion of the probes in a given cell type, and (c) the membrane fluidity of normal and neoplastic mast cells. The effect of compound 48/80 has also been examined.

METHODS

Spin-labeled probes and 48/80. Compound 48/80 was synthesized according to the procedure of Baltzly *et al.* [6]. All of the spin-labeled probes were purchased from Syva Associates, Palo Alto, CA.

Cell preparation. Peritoneal and pleural cavity cells were obtained by lavage as described previously [7]. The resultant cell suspension was filtered through monofilament nylon cloth (43 μm^2 New York Stensil

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† Abbreviations employed: e.s.r., electron spin resonance; 5-DSA, 5-doxylstearic acid; 12-DSA, 12-doxylstearic acid; 16-DSA, 16-doxylstearic acid; 5-DMS, 5-doxylmethylstearate; 12-DMS, 12-doxylmethylstearate; and 16-DMS, 16-doxylmethylstearate.

Silk Corp. New York, NY), to remove clumps of dead cells and debris, and then was concentrated by centrifugation.

Mast cells were purified by centrifugal elutriation using a Beckman J-21C centrifuge (Beckman Instruments, Norcross, GA) equipped with a JE-6 Elutriator rotor, cooled to 4°. A silicone agent (Sigmacote SL-2, Sigma Chemical Corp., St. Louis, MO) was applied to the separation chamber. Locke's solution containing 0.1% bovine serum albumin (Sigma Chemical Corp., crystallized) was chilled in an ice bath prior to use. The peritoneal and pleural cells were concentrated to 25 ml and placed in the sample mixing chamber which was then attached to the flow system. All of the air was expelled by introducing Locke's solution through the injection valve. Additional bubbles trapped in the rotor were removed by hand spinning the rotor until no back pressure was registered on the gauge. The cells were loaded into the separation chamber at 2000 rpm and 20 ml/min. After 200 ml had been collected (10 min), the sample mixing chamber was bypassed, the flow was decreased to 15 ml/min, and the rotor speed was reduced to 1500 ± 20 rpm. A final 200 ml of fluid was collected under these conditions. Flow was stopped at this point to allow the turbulence to cease and to permit the remaining cells to pellet. The pellet was suspended in 5 ml of Locke's solution (without albumin) and removed using a Pasteur pipette. The cells were washed once in Lock's solution to remove any residual albumin. The purity ranged from 61 to 89 per cent (average = 71 per cent) for fluids with 2–10 per cent (average = 6 per cent) mast cell concentrations; total purification time was a maximum of 25 min.

Mastocytoma cells from an established P-815 ascites cell line [8] were grown in CD_2F-1 mice (Charles River) and harvested on day 5 or 6 after inoculation. They were washed three times in Locke's solution before use.

Spectral measurements. Electron spin resonance spectra were recorded using a Varian E-109 X-band spectrometer equipped with a TM_{110} cavity. The large dimensions of this cavity allowed the use of relatively high power levels without saturation. A significant increase in temperature inside the flat cell was not observed during the time frame of these experiments. The instrumental parameters were as follows: scan range, 100 G; field set, 3350 G; time constant, 0.5 sec; scan time, 4 min; modulation amplitude, 4 G; modulation frequency, 100 kHz; receiver gain, 3.2×10^4; microwave power, 30 mW; and microwave frequency, 9.5 GHz. All spectra were recorded at room temperature using a large, quartz, aqueous-sample, flat cell.

The stock spin-label solutions were made up in methanol at a concentration of 1×10^{-3}M. The cells were labeled by adding the stock solution to fresh cells at dilutions from 1:100 to 1:500. No difference was observed when the cells were labeled by incubating them with spin-label solution that had been evaporated onto a glass surface. The spectra were recorded immediately, as signal decay proceeds rapidly in living cells. The spin-labeled cells did not release histamine, appeared normal in all respects under the microscope, and excluded trypan blue.

After the control spectra had been recorded, the cells were removed from the cavity, 48/80 was added (10 μg/ml), and the e.s.r. signal was recorded. Mast cells from twenty rats, concentrated to 500 μl, were needed for each spectrum. After 48/80 treatment, microscopic examination of these cells showed massive degranulation of a majority of the cells; trypan blue was excluded, however, indicating that the cells were still viable.

The effect of 48/80 on the environment of membrane-bound 5-DSA and 5-DMS (Fig. 3) was determined using the method of Mason *et al.* [9], in which the half-width of the low field hyperfine extremum of the probes was measured at half its height. This method is very sensitive to small changes in the e.s.r. spectral characteristics.

The rotational correlation time, τ (sec), of the membrane bound spin labels, was calculated from the first derivative presentation using the following formula:

$$\tau = 6.5 \times 10^{-10} W_0 \left[\left(\frac{I_0}{I_1} \right)^{\frac{1}{2}} - 1 \right] \qquad (1)$$

where W_0 is the linewidth of the central peak and I_0 and I_1 are the relative intensities of the central peak and the high field extremum respectively [10].

The S formalism for determining the order parameter was derived using the following equation:

$$S = \frac{T_{\parallel} - T_{\perp}}{3(T_{zz} - a')/2} \qquad (2)$$

where $T_{\parallel}$ and $T_{\perp}$ are defined in Fig. 2, the tensor T_{zz} = 32 G and $a' = (T'_{\parallel} + 2T'_{\perp})/3$ [11].

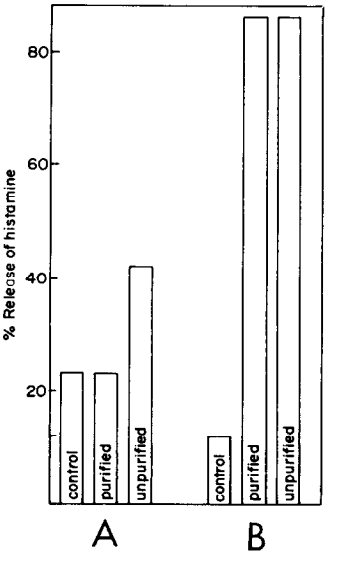

Fig. 1. Effect of albumin on histamine release by mast cells after centrifugal elutriation. Mast cells were elutriated in Locke's solution with and without bovine serum albumin (0.1%). Unpurified cells from the same batch were maintained in the same solution during elutriation. The concentration of 48/80 was 1.0 μg/ml. A: cells elutriated in Locke's solution; B: cells elutriated in Locke's solution plus albumin.

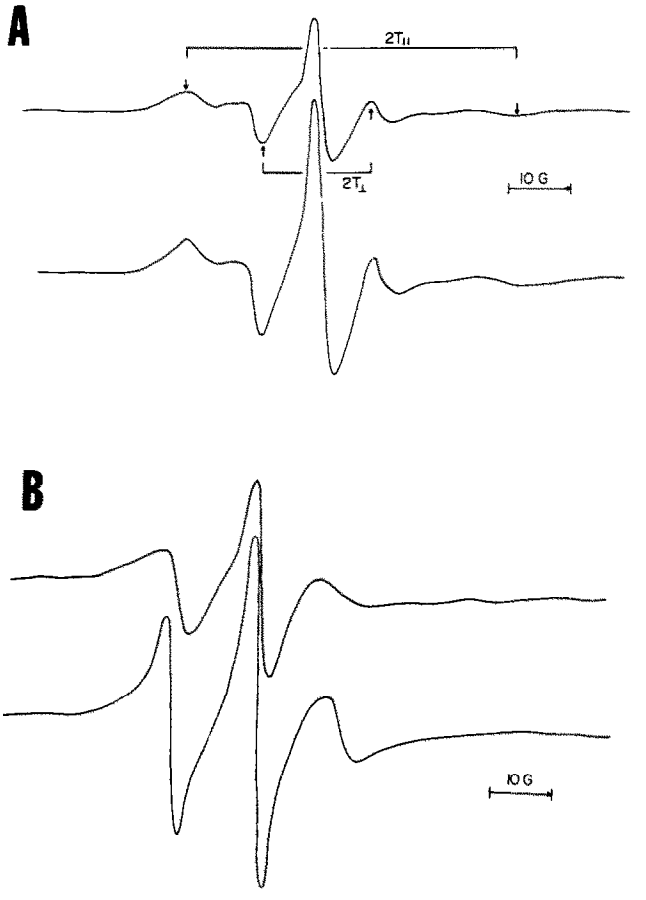

Fig. 2. Electron spin resonance spectra of 5-doxyl methyl stearate and 5-doxyl stearic acid in purified mast cells and in mastocytoma cells. The cells were labeled (5×10^{-5} M) and the spectrum was recorded immediately. A: 5-doxyl stearic acid; B: 5-doxyl methyl stearate. In each case the upper spectrum is from purified mast cells.

Spectra that could not be described by using either τ or S were arbitrarily characterized by comparing the relative amplitudes of the low field and central peaks (Table 1).

RESULTS

Glick *et al.* [12] have shown previously that centrifugal elutriation could be used for the isolation of mast cells; they did not, however, report the histamine releasing ability of the purified cells. This was determined in the present study by comparing elutriated cells with unprocessed cells from the same stock (Fig. 1). Cells that had been processed without albumin were visibly damaged and exhibited high spontaneous histamine release. They did not respond at all to compound 48/80 (including concentrations as high as 10 μg/ml). Unpurified mast cells stored without albumin were weakly responsive to 48/80. When peritoneal cells were elutriated in the presence of albumin, the purified mast cells were highly responsive to 48/80 and showed no visible damage. Elutriated mast cells released histamine in a manner similar to the release by cells stored in the presence of albumin (Fig. 1).

The e.s.r. spectra of 5-DMS and 5-DSA in both mast cells and mastocytoma cells are shown in Fig. 2. The spectra suggest that 5-DMS was more highly immobilized in the mast cells than in the mastocytoma cells. In contrast, 5-DSA was relatively more highly immobilized in both cell types and there was no apparent difference between them. Rotational correlation times for some of the labels are presented in Table 1. The motion of the nitroxide group attached to stearic acid was very dependent on the position of the nitroxide moiety with respect to the polar end of the molecule. Specifically, there was a progressive increase in the motion of the nitroxide as it was located further from the ionized carboxyl group and, presumably, further into the hydrophobic region of the cell. The signals from spin-labeled methyl stearate analogs showed a similar pattern. Their motion, however, was not as strongly influenced by the position of the nitroxide. Although the order parameter (S) for 5-DSA was the same in both mastocytoma cells and mast cells, the other labels were more immobilized in the mast cells. Further, in each case the nitroxide attached to the acid was more highly immobilized than its counterpart attached to the ester; this difference, however,

Table 1. Rotational correlation times and peak ratios for spin labels in mastocytoma cells and mast cells and the effect of 48/80

	τ (nsec)		Ratio*		Change in fluidity after 48/80 (10 μg/ml)	
	Mastocytoma	Mast cells	Mastocytoma	Mast cells	Mastocytoma	Mast cells
5-DSA	†	†	†	†	−1.6‡	0§
5-DMS	0.29	†	1.39	2.33	−0.09	0.8‖
12-DSA	0.84	†	2.11	4.76	+0.06	ND¶
12-DMS	0.18	0.78	1.21	1.93	+0.05	ND¶
16-DSA	0.24	0.30	1.35	1.48	−0.03	−2.9‖
16-DMS	0.09	0.31	1.17	1.52	−0.05	+9.2‖

* Ratio of the amplitude of the center peak to the low field peak.
† These parameters could not be calculated due to the spectral line shapes (see text).
§ Per cent change in the order parameter S.
‡ Change in τ (nsec).
‖ Percent change in the peak ratios.
¶ Not determined.

decreased as the nitroxide group was moved further from the charged end of the molecule.

The correlation times derived from spin labels in mast cell-depleted leukocytes indicate that mast cell membranes were similar in fluidity to the other assorted white blood cells found in the rat peritoneal cavity.

The effect of 48/80 on stearic acid and ester spin labels incorporated into either mast cells or mastocytoma cells is shown in Table 1. The apparent molecular motion of the spin labels was not significantly affected by 48/80 even at a concentration far exceeding that which was maximally active. The method of Mason *et al.* [9] was used to measure the influence of spin-label concentration in the membrane on the e.s.r. spectrum of the nitroxide. The data show that, when the cells were incubated with spin-label concentrations higher than 2.5×10^{-5}M, the e.s.r. extrema of the membrane-bound labels were broadened, presumably due to magnetic interactions between closely adjacent nitroxide molecules [13] Figure 3 shows the effect of 48/80 on various concentrations of spin label within the cell membrane. Since the cell concentration varied somewhat between experiments, only spectra that displayed intermolecular exchange broadening were used. The data show that the effect of 48/80 on the spectra was apparent only in cases where there were magnetic interactions present. The presence of 48/80 in these experiments reduced the magnetic interactions, and the spectra resumed a normal shape. There was no effect of 48/80 on labeled cells in which the membrane concentration of the probe was magnetically dilute.

DISCUSSION

The e.s.r. spectrum of a rapidly tumbling nitroxide reflects its rotational correlation time, τ. Measurements of τ, therefore, are useful in quantifying the constraint on the motion of the nitroxide produced by its immediate environment, and are easily calculated from the first derivative e.s.r. spectrum using equation 1. The use of this equation, however, is only valid when molecular ordering does not interfere with the line shape of the spectrum. In biological membranes, stearic acid and ester probes assume a specific orientation dictated by fatty acid side chains of the phospholipids, which are perpendicular to the membrane surface [11]. The resulting anisotropy can

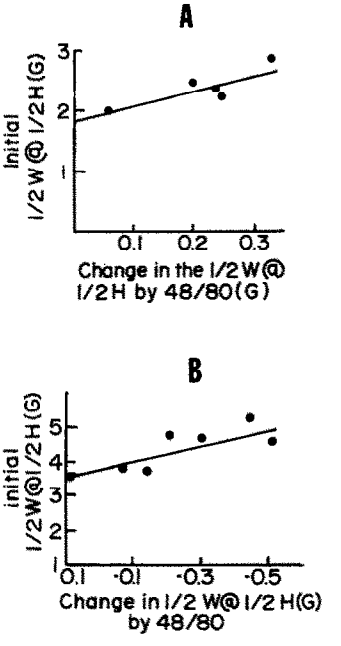

Fig. 3. Effect of 48/80 on the width of the high field hyperfine extrema of 5-DMS and 5-DSA in mastocytoma cells (10^7 cells/ml). The spectra were recorded at various concentrations. The presence of magnetic interactions was determined by the increasing width of the high field extrema as represented on the ordinate (1/2 w at 1/2 h, 1/2 the width measured at 1/2 the height, reported in Gauss). After the control spectra were taken, 48/80 (100 μg/ml) was added and the spectrum recorded immediately. A: 5-DMS; B: 5-DSA.

become sufficient to contribute line broadening effects that are indistinguishable from motional line broadening. Therefore, calculation of τ under these conditions is invalid. The spectra of 16-DMS, 16-DSA and 12-DMS in both mast cells and mast cell-depleted cells and of 16-DMS, 16-DSA, 12-DMS, 12-DSA and 5-DMS in mastocytoma cells were characterized by Lorentzian line shapes. Since this suggests that the correlation time (τ) was rapid and that the spectra were not influenced significantly by angular dependence, τ can be calculated using equation 1. The e.s.r. spectra of 12-DSA, 5-DMS and 5-DSA in mast cells and in mast cell-depleted cells and that of 5-DSA in mastocytoma cells, however, were influenced by the molecular order imposed on the nitroxide by its environment. Since the order parameter, S, in similar systems is related to motion, we have calculated the S value for 5-DSA both in mast cells and in mastocytoma cells, using equation 2. The spectra of 12-DSA and 5-DMS in mast cells and in mast cell-depleted cells were intermediate in that neither the S value nor τ calculation could be derived. In these cases, a ratio was determined from the relative amplitudes of the low field and central peaks. Although this parameter is not valid in an absolute sense for measuring rotational correlation times or order parameters, it is related to the calculated values of τ (where these could be made). For the purposes of this study, it suffices only to extend approximate fluidity comparisons into motional regions in which τ could not be calculated.

The stearic acid and methyl stearate spin labels intercalate in the plasma membrane bilayer in the region near the glycerol backbone of the membrane phospholipids. The polar part of the labels is in contact with the more hydrophilic interface region around the glycerol backbone, which is also populated by the hydrophilic portions of the membrane proteins. The interface and the more hydrophobic areas of cell membranes can be studied by observing the motion of a nitroxide reporter group at different points along the fatty acid or ester chain. This study and others [14] have shown that the motion of the nitroxide increases as the reporter group is located further into the hydrocarbon region of the membrane. This finding is consistent with the current theories regarding membrane structure in that the most molecular ordering occurs at the surface and interface regions, presumably due to the presence of peripheral proteins and the glycerol backbone of phospholipids. Nuclear magnetic resonance studies have shown that the molecular ordering of membrane probes decreases abruptly at a certain point toward the hydrophobic interior of the membrane [14]. Both in mast cells and in mastocytoma cells, the molecular ordering as reported by the 5-DSA was similar. The motion of the nitroxide, however, increased very rapidly in the mastocytoma cells as reported by the signal from the 12th carbon, and there is little change from the 12th and the 16th carbon. In the mast cells, the increase in fluidity was more gradual and the abrupt change came between the 12th and the 16th carbon atoms. Although this effect was more pronounced with the acid derivatives, the methyl esters also showed a similar pattern. Although this finding indicates a fundamental difference between the mast cell and the mastocytoma cell membrane, its significance, if any, in explaining the neoplastic character of the mastocytoma cells is unclear.

The difference in reported fluidity seen between the acid and ester derivatives was greatest when the nitroxide was closest to the acid or ester group (5-DMS, 5-DSA), and diminished as it was situated further away (16-DMS, 16-DSA). This was seen with both mast cells and mastocytoma cells and is probably due to the stronger "anchoring" effect of the acid moiety. In a preliminary study [5], we reported that mast cell and mastocytoma cell membranes were similar as indicated by 5-DSA. This was in contrast to the large difference observed with the fluorescent hydrophobic probe, diphenylhexatriene [16]. In the present study, the 5-DMS probe suggests that mast cell membranes are indeed more rigid at the interface; furthermore, the other probes show that this difference persists into the hydrophobic region of the membrane. This study emphasizes that the use of 5-doxyl stearic acid to compare different membrane types can lead to erroneous interpretations, due to the strong influence of the charged group anchoring the molecule. Therefore, several different probes should always be included in a complete study of membrane structure.

The fluidity of the mast cell membrane was similar to that found in other leukocytes from the rat peritoneal and pleural cavities. Thus, the ability of the rat peritoneal mast cell to secrete large numbers of granules within a few seconds of stimulation is not related to a fundamental difference in this aspect of its membrane structure. Kennerly *et al.* [17] have demonstrated a substantial alteration in the composition of mast cell membrane phospholipids after histamine secretion stimulated by various agents. The data indicated that membrane stimulation initially resulted in an increase in 1,2-diacyglycerol, which is then converted to other phospholipid species. These authors have suggested that such alterations in phospholipid composition may modulate the activity of membrane enzymes as a result of membrane fluidity changes. Although these changes in phospholipid composition were demonstrated well within the time scale of our e.s.r. measurements, it was not possible to demonstrate any effect of compound 48/80 on mast cell stearic acid and ester probes that were magnetically dilute. Spin-labeled stearic acids and esters have been used successfully to demonstrate membrane fluidity changes in response to temperature [18], divalent cations [19], and drugs [20]. Therefore, the absence of any effect by 48/80 on these labels suggests strongly that the action of this agent does not result in a permanent major fluidity change among the membrane lipids or at the interface. A rapid, transient change in fluidity, however, would not have been detected using this technique. Recently, Merritt and Loughman [21] have reported a fluidity decrease in mastocytoma cells after immunological challenge. This change coincided with histamine release and was dependent on temperature, time, and dosage. In contrast, we have not seen a consistent, measurable change in the motional characteristics of any of our probes in either mastocytoma cells or in purified

mast cells after histamine secretion stimulated with 48/80. These findings may signify a basic difference between the secretory response initiated by chemical means and the immunological reaction.

The effect of 48/80 on high concentrations of 5-DMS and 5-DSA in mastocytoma cell membranes indicates that 48/80 increased the distance between interacting spin-labeled molecules. One possible explanation for the reduced intermolecular magnetic interactions is that compound 48/80 increased the membrane area available for the intercalation of the spin labels. We have shown previously that 48/80 causes changes both in intact cells and in isolated cell membranes which may be due to denaturation and aggregation of membrane proteins [22]. We have also shown that 48/80 treatment of both normal and neoplastic mast cells increases the binding of 1-anilinoaphthalene-8-sulfonate, and other charged molecules including 48/80 itself (unpublished results). Therefore, although 48/80 does not cause a permanent fluidity change, it does alter the state of the membrane, giving rise to additional binding sites for charged molecules of diverse structure. If 48/80 acts by causing a partial denaturation of the membrane proteins [22], the disruption may affect the ordering of phospholipids, resulting in a wider spacing between intercalated spin labels. In addition, treatment with 48/80 may also allow charged compounds to translocate more rapidly across the membrane bilayer and bind to the inner side.

In conclusion, we have shown that through the use of centrifugal elutriation, viable purified mast cells can be obtained rapidly in quantities sufficient for spin-label studies. Mast cell membrane fluidity was similar to that of the other peritoneal cells; however, with the exception of 5-DSA, all of the probes reported a greater fluidity in the mastocytoma cell membrane. This was consistent with our previous findings using fluorescent probes. We have also shown that compound 48/80 does not permanently influence the fluidity of mast cells or mastocytoma cells in any of the membrane regions studied. Treatment with 48/80 did, however, decrease probe–probe intermolecular interactions among stearic acid and ester probes in mastocytoma cell membranes. This finding is consistent with our previous observations that 48/80 increases the membrane area available for charged molecules.

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