RAMAN SPECTROSCOPY OF NERVE FIBERS

A Study of Membrane Lipids under Steady State Conditions

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ABSTRACT The molecular structures of different nerve fibers kept in good physiological conditions were studied by laser Raman spectroscopy. For myelinated nerves like the rat sciatic nerve, the Raman spectrum is dominated by bands due to the lipid component of the myelin sheath. The temperature dependence of these bands does not reveal any thermotropic phase transition between 0 and 40°C. There is, however, with temperature, a linear increase in the intermolecular disorder that is accompanied by an increase in the number of *gauche* bonds of the phospholipid acyl chains. For unmyelinated nerves such as the lobster leg nerve, the C-H stretching region of the Raman spectrum is covered by bands arising from the protein component of the axoplasm. However, for the garfish olfactory nerve that has a high density of excitable membranes, phospholipid bands are observed and can be used as intrinsic structural probes of the excitable membranes. The relative intensity of these bands is also temperature dependent.

INTRODUCTION

The concept of the fluid mosaic model of the structure of biological membranes (Singer and Nicolson, 1972) has stimulated many experiments and speculations about the role of membrane fluidity in explaining various aspects of membrane physiology and functions (for a review see Kimelberg, 1977). At the nerve level, it has been suggested that the membrane fluidity plays a likely role in the anesthetic action (Lee, 1976) and, more generally, in the molecular basis of excitability (Keynes, 1972; Hille, 1978), but experimental evidence is still scanty.

Nerve membrane fluidity was first investigated by Hubbell and McConnell using spin-labeled fatty acids, and they demonstrated that the motion of the aliphatic chains in the axonal membranes of *Homarus americanus* is very similar to that of a 2:1 egg phosphatidylcholine/cholesterol mixture (Hubbell and McConnell, 1969, 1971). The same technique has also been used to investigate the thermotropic behavior of the myelin membrane of rat and frog sciatic nerves (Schummer et al., 1975) and of normal and pathological human peripheral nerves (Laporte et al., 1979).

More recently, different nerve preparations, labeled with the fluidity-dependent fluorescent probe pyrene, have shown under steady-state conditions a temperature-dependent increase of membrane fluidity very similar to that of the extracted lipids (Georgescauld et al., 1979). When stimulated, the pyrene-labeled axonal membranes showed a transient variation of the pyrene fluorescence, which was interpreted as a possible transient decrease of fluidity of the nerve-membrane lipids during excitation (Georgescauld and Duclohier, 1978).

Both fluorescence and spin-label techniques require the use of external probes that, at the usual concentration of one to five probe molecules per hundred lipids, induce significant perturbations of the physiological functions of the excitable membrane and shorten the lifetime of the nerve preparations (Duclohier et al., 1980). One way to avoid these problems is to use Raman spectroscopy, which provides intrinsic molecular information without using external probes.

During the last decade, Raman spectroscopy has been used successfully to study the conformation of phospholipids in model and natural membranes (Wallach et al., 1979; Carey, 1982; Tu, 1982). Raman bands are sensitive to the strength of the chemical bonds and to the geometry of the scattering molecules. For example, the C-C and C-H stretching bands in the Raman spectra of lipid bilayer systems are remarkably sensitive to the conformation of the hydrocarbon chains and have been used frequently to monitor conformational disorder in these systems. Furthermore, since Raman spectroscopy is a noninvasive technique, it has also been used to study the structure of intact living tissue such as muscle fibers (Pézolet et al., 1980; Caillé et al., 1983).

Raman spectra of myelinated frog sciatic nerves have already been published (Larsson and Rand, 1973; Szalontai et al., 1977), but because of the presence of carotenoids in this type of fiber, little information regarding the myelin sheath has been obtained. In addition, the myelin sheath is not directly involved in the transmission of the nerve

impulse and only some nonmyelinated nerve trunks, such as garfish or pike olfactory nerves, contain a high percentage of conducting nerve membranes.

Here we present the first Raman spectra of unmyelinated, physiologically active nerve fibers and spectra of the myelin sheath of the rat sciatic nerve. The effect of temperature on these spectra demonstrates clearly that Raman spectroscopy can be used successfully to obtain information on the conformation of the axonal membrane lipids or of the myelin sheath of nerve fibers.

MATERIAL AND METHODS

Nerve Preparations

Throughout this study, we have used three different types of nerves: the sciatic nerve of the rat, the leg nerve of the lobster *Homarus americanus*, and the olfactory nerve of the garfish *Lepisosteus trichosteus*. After dissection, the nerve trunks were ligated at both ends. The composition of the physiological solutions in millimoles per liter were the following: (*a*) artificial sea water for the lobster nerve: NaCl, 400; $CaCl_2$, 11; $MgCl_2$, 55; Tris, 5 (pH 7.4); (*b*) Loke's solution for the rat sciatic nerve: NaCl, 154; KCl, 5.6; $CaCl_2$, 2.2; $MgCl_2$, 1; dextrose, 5; Tris, 8 (pH 7.4); (*c*) Ringer's solution for the garfish olfactory nerve: NaCl, 120; KCl, 4; $CaCl_2$, 10; dextrose, 60; glucose, 24; $NaHCO_3$, 3 (pH 6.4). Routinely, before starting the Raman measurements, the nerves were tested externally with a conventional electrophysiological device, and nonfunctional nerves were discarded.

Nerve fibers were inserted into glass capillary tubes of the desired length and of 0.8 mm i.d. The appropriate physiological solution was then introduced into the capillary, which was sealed with hematocrit tube sealant. Throughout the experiments, the temperature was controlled to within 0.1°C by means of a water-jacketed capillary cell holder.

To check the effect on electrophysiological activity of prolonged exposure of the nerve fibers to the laser beam, we have designed a Raman cell fitted with platinum electrodes for external stimulation and recording of action potentials. With this apparatus, we have been able to verify that at 5°C, the functionality of the nerve preparations was not affected by the low-power laser beam for several hours of irradiation. Therefore, the samples used were electrophysiologically active at least at low temperature, even after exposure to the laser beam.

Protein and Phospholipid

Bovine serum albumin (BSA) was obtained from Sigma Chemical Co. (St. Louis, MO). Egg yolk phosphatidylcholine (EPC) was purified in our laboratory according to the method described by Singleton et al. (1965). A 10% by weight aqueous dispersion of EPC was prepared by shaking mechanically at 30°C the phospholipid in water at pH 7.5. The sample was then transferred into a Raman capillary cell (1.5 mm diam) and centrifuged to yield white pellets used to obtain the spectra.

Raman Spectroscopy

The Raman spectra were excited with the 488.0 nm line of an argon ion laser (model 164; Spectra-Physics Inc., Mountain View, CA) and measured with a Coderg PHO double monochromator equipped with a cooled photomultiplier tube (C1034A; RCA, Solid State Division, Lancaster, PA) and photon counting detection. The monochromator was calibrated with discharge lamps and the frequency shifts cited later are believed to be accurate to $\pm 2\ cm^{-1}$ for sharp peaks. The spectral slitwidth was 6 cm^{-1} and the laser power at the sample was between 100 and 200 mW. At this low power, we have not noticed any sample degradation even for experiments involving prolonged heating. The incident laser beam was aligned perpendicularly to the capillary axis and the scattered light was collected at a right angle to the incoming radiation. Raman spectra were acquired with a minicomputer (LSI 11 PDP 11/03; Digital Equipment Corp., Marlboro, MA) that allows for multiple scanning and various forms of data treatment.

RESULTS AND DISCUSSION

Assignment of Nerve Fiber Raman Spectra

Raman spectra in the C-H stretching region of nerve fibers from three different sources are reproduced in Fig. 1. For comparison the spectra of a natural phospholipid, EPC, and of a protein, BSA, are shown in Fig. 2. As for pure phospholipids, the Raman spectra of nerve fibers in the C-H region are characterized by three unresolved bands at ~2,850, 2,885, and 2,940 cm^{-1}. The first two bands are respectively assigned to the methylene symmetric and antisymmetric C-H stretching modes of the phospholipid acyl chains (Gaber and Peticolas, 1977; Bunow and Levin, 1977), while the 2,940 cm^{-1} band results in part from underlying infrared active methylene asymmetric stretching modes that become Raman active when the intramolecular chain disorder is increased (Bunow and Levin, 1977). As shown in Fig. 2, the protein component of the membranes and of the axoplasm of nerve fibers may also contribute to the scattering intensity at ~2,930 cm^{-1}.

The comparison of Figs. 1 and 2 reveals that the Raman

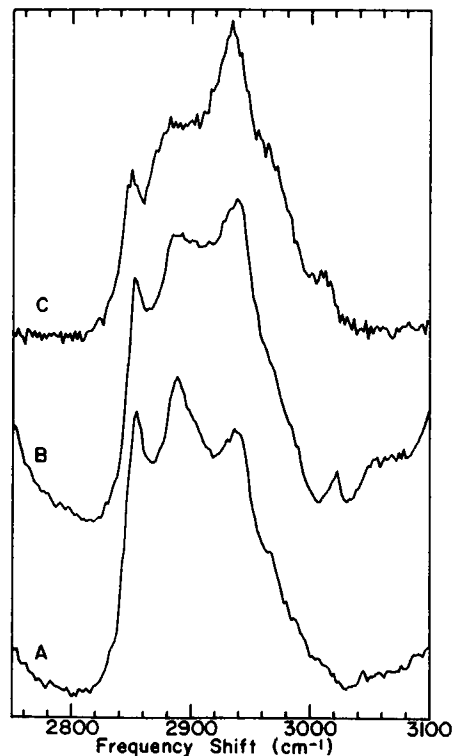

FIGURE 1 This figure shows Raman spectra of the C-H stretching region of intact rat sciatic nerve at 20°C (*A*), garfish olfactory nerve at 6°C (*B*), and lobster leg nerve at 15°C (*C*). Spectra *A* and *B* are averages of four scans at 1 s/2 cm^{-1} step, whereas spectrum *C* is the average of nine scans at 1 s/2 cm^{-1} step.

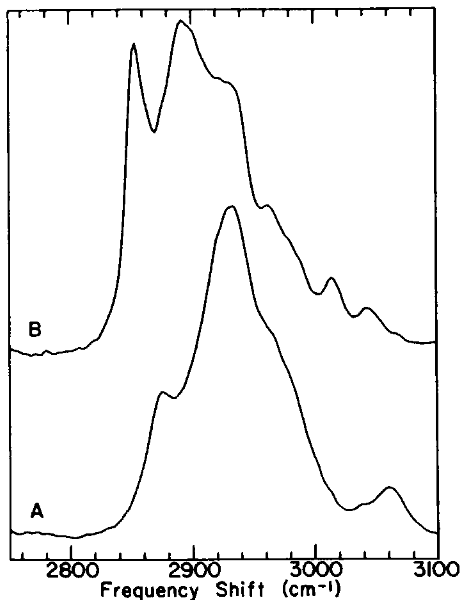

FIGURE 2 This figure shows Raman spectra at 20°C of the C-H stretching region of solid bovine serum albumin (*A*) and of an egg phosphatidylcholine dispersion in water (*B*). Each spectrum is an average of four scans at 1 s/2 cm^{-1} step.

spectrum of the myelinated rat sciatic nerve is very similar to the EPC spectrum. Thus, for such fibers, the spectrum is dominated by the contribution of the lipid component of the myelin sheath, which has a protein content of only 20%. On the contrary, the spectrum of the unmyelinated lobster leg nerve displays a strong protein band at 2,940 cm^{-1}, with only a weak phospholipid contribution at 2,850 cm^{-1}. Therefore, because of the low membrane density of this type of fiber, little information can be obtained on the phospholipid structure. For the unmyelinated garfish olfactory nerve, which has a high density of excitable membranes (Easton, 1971), the phospholipid bands are well resolved from the protein band at 2,930 cm^{-1} and can thus be used as intrinsic probes of the structure of phospholipids of the excitable membranes.

The Raman spectrum between 700 and 1,900 cm^{-1} of the rat sciatic nerve is shown in Fig. 3. As for the C-H stretching region, the major bands in this region come from the lipid component of the myelin membrane. For example, the C=C stretching mode of the unsaturated acyl chains gives a strong band at 1,670 cm^{-1} that completely masks the protein's amide I region. The carbonyl C=0 stretching mode appears at 1,745 cm^{-1}, whereas the CH_2 bending and twisting modes give bands at 1,465, 1,445, and 1,302 cm^{-1}, respectively.

The C-C skeletal stretching mode region between 1,000 and 1,200 cm^{-1} is of greatest lipid structural interest since it provides direct information on the intramolecular order of the phospholipid aliphatic chains (Yellin and Levin, 1977; Wallach et al., 1979). In the case of the rat sciatic nerve, the C-C region displays two bands at 1,067 and 1,132 cm^{-1} that are characteristic of the *trans* conforma-

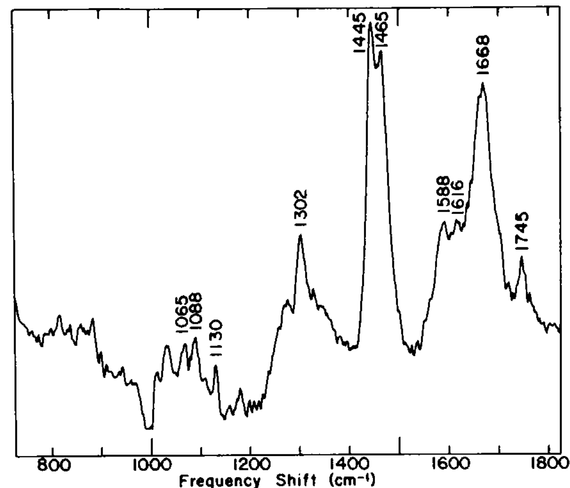

FIGURE 3 This figure shows a Raman spectrum at 20°C of the 700–1,900 cm^{-1} region of an intact rat sciatic nerve. This spectrum is an average of six scans at 1 s/2 cm^{-1} step.

tion, while the 1,090 cm^{-1} band is assigned to the presence of *gauche* conformers.

Spectra in the 700–1,900 cm^{-1} region were also recorded for the crab and garfish nerves, but no valuable information was obtained from them since they were completely dominated by strong resonance Raman bands of the carotenoid pigments present in these samples (Larsson and Rand, 1973). Similar carotenoid problems were

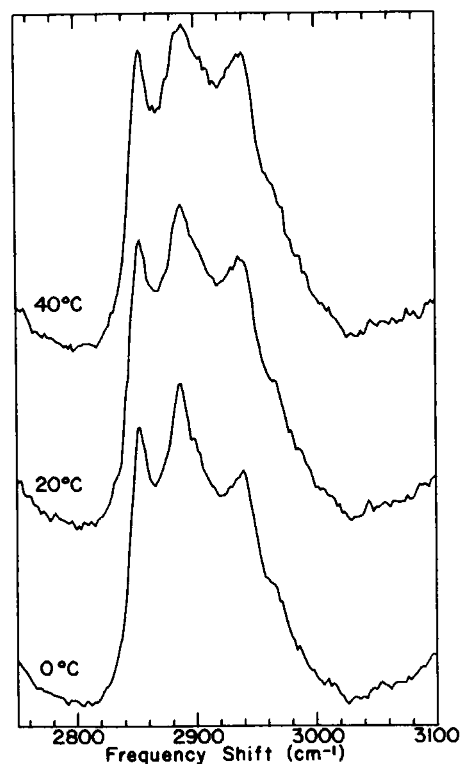

FIGURE 4 The effect of temperature on the C-H stretching region of the Raman spectrum of a rat sciatic nerve is shown. The conditions are the same as those in Fig. 1.

also encountered with the frog sciatic nerve. In fact, we have found that it is very difficult to obtain reproducible results with this type of fiber since the presence of strong absorbing species causes overheating and degradation of the fibers in the laser beam.

Temperature Dependence of Nerve Spectra

Since it is well known that Raman spectra of phospholipid bilayers are sensitive to the acyl chain intra- and intermolecular disorder, we have studied the temperature dependence of the Raman spectra of the rat sciatic nerve and of the garfish olfactory nerve.

Fig. 4 shows the effect of temperature on the C-H stretching region of the Raman spectrum of the sciatic nerve. As the temperature is increased, the width of the 2,885 cm^{-1} band increases, indicating an increase of the rotational mobility of the phospholipid acyl chains (Snyder et al., 1980). Simultaneously, the intensity of the 2,940 cm^{-1} band increases as the system becomes more disordered. Note that although the proteins contribute to the intensity at ~2,930 cm^{-1}, they cannot account for the observed spectral change when the temperature is increased since the C-H stretching region of the Raman spectra of proteins is not very sensitive to temperature. For example, we have observed that, within the experimental error, the 2,750–3,100 cm^{-1} region of the spectrum of BSA in aqueous solution does not change between 10 and 40°C.

Fig. 5 displays the temperature profile based on the peak height intensities of the bands at 2,940 and 2,885 cm^{-1}. Increase of the intensity of these two bands reflects an increase of the intermolecular chain disorder and order, respectively (Bunow and Levin, 1977). As seen in Fig. 5, temperature induces a significant chain disordering of the phospholipid molecules of the myelin membrane. The $h_{2,940}/h_{2,885}$ ratio increases linearly with temperature and no thermotropic transition is detected within the examined temperature range, a finding that is in good agreement with the spin-label study of Schummer et al. (1975) on the rat sciatic nerve. A phase transition for the central nervous system myelin was also not detected using conventional calorimetry (Ladbrooke et al., 1968) but more recently, thermotropic phase transitions were observed for normal human myelin using a high-sensitivity microcalorimeter (Moscarello et al., 1983). These transitions were assigned to the melting of lipid-protein complexes since they were not observed for the total myelin lipid or protein extracts. The apparent discrepancy between our Raman results and the calorimetric ones may be accounted for by differences in composition and structure of the myelin used or because the Raman intensity ratio used throughout this study is mainly sensitive to lipid hydrocarbon intermolecular interactions.

To determine the intra- and intermolecular contribution of the phospholipid disordering induced by temperature, we have also studied the effect of temperature on the C-C stretching region of the rat sciatic nerve. Even though the bands are weak in this region, Fig. 6 demonstrates that the number of *gauche* bonds increases significantly with temperature as detected by the increase of the 1,080 cm^{-1} band. The $h_{1,130}/h_{1,080}$ intensity ratio, which is related to the ratio of the *trans* and *gauche* conformer populations, decreases from 0.78 at 0°C to 0.56 at 40°C. Note that at 25°C, the $h_{1,130}/h_{1,080}$ ratio for the rat sciatic nerve (0.70), which has a cholesterol content of 40% (Schummer et al., 1975), is much higher than the value found for EPC (0.16) but is closer to the 0.58 ratio observed for EPC/cholesterol at a 1:1 molar ratio (Mendelsohn, 1972). This is also in agreement with the results obtained by infrared spectroscopy on myelin, which indicate that there is a considerable

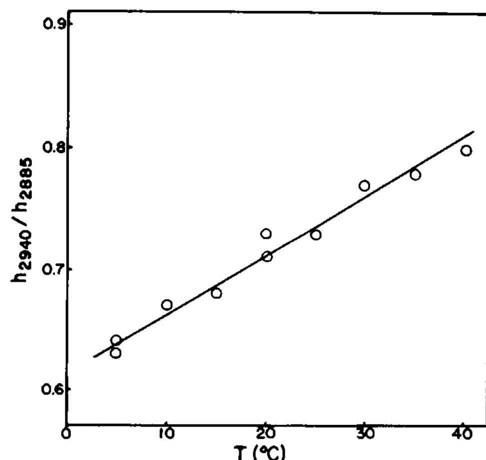

FIGURE 5 The $h_{2,940}/h_{2,885}$ intensity ratio as a function of temperature for a rat sciatic nerve is shown.

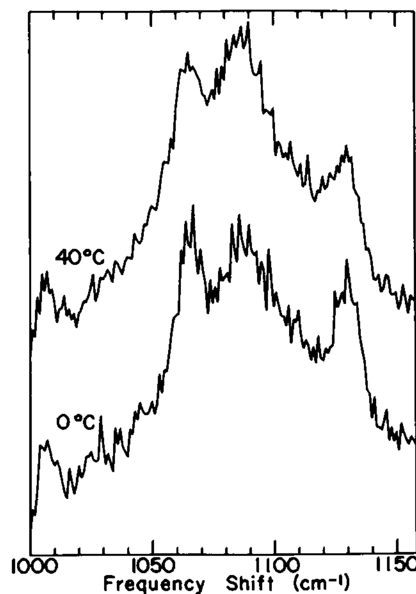

FIGURE 6 The effect of temperature on the C-C stretching region of the Raman spectrum of a rat sciatic nerve is shown. Each spectrum is an average of 22 scans at 1 s/cm^{-1} step.

degree of planar *trans*-configuration for the lipid chains within the membrane structure (Jenkinson et al., 1969). Therefore, the temperature-induced chain disordering detected from the C-H stretching region is partly due to intramolecular conformational changes of the phospholipid acyl chains.

The effect of temperature on the Raman spectrum of the unmyelinated garfish olfactory nerve is shown in Fig. 7. Even though the phospholipid bands are weaker than in the spectra of myelinated nerves, it can be seen that temperature induces an intermolecular disordering of the lipid chains of the excitable membrane of the nerve fiber. As for the sciatic nerve, the $h_{2,940}/h_{2,885}$ intensity ratio increases linearly with temperature from 1.09 at 6°C to 1.22 at 25°C. No phase transition has been observed between 0 and 30°C. The percent variation of $h_{2,940}/h_{2,885}$ is $\sim$15% from 5 to 25°C, in spite of the lower cholesterol content of the garfish nerve (Chacko et al., 1976). Presumably, the higher protein content of the latter nerve interferes with the measurement of the lipid C-H bands. This suggestion is supported by the higher value of the $h_{2,940}/h_{2,885}$ ratio for the garfish nerve. For natural membranes such as the sarcoplasmic reticulum, the protein content is so high that no effect has been observed on the C-H region of the Raman spectrum of this membrane (Lippert et al., 1981).

In conclusion, our results show that Raman spectroscopy can be used to obtain information on the structure of membrane lipids of nerve fibers even for unmyelinated nerves such as the garfish olfactory nerve. They are in good agreement with previous results obtained using fluidity dependent fluorescent probes such as pyrene (Georgescauld et al., 1979).

During the past fifteen years, several spectroscopic methods have been used with the hope of obtaining structural information at the membrane level of nerve fibers. Intrinsic optical signals such as birefringence (Von Muralt et al., 1976) suggest some conformational changes during the action potential but do not provide enough details at the molecular level. On the other hand, in spite of the wide use of fluorescence probes (Cohen et al., 1974), the results are not directly interpretable because the distribution of the probes between the different membrane components is not well understood.

The results of this paper show that a noninvasive and intrinsic probe such as Raman spectroscopy represents a useful tool for the detection of specific conformational changes in nerve fibers, including changes that may be related to physiological functions or pharmacological effects.

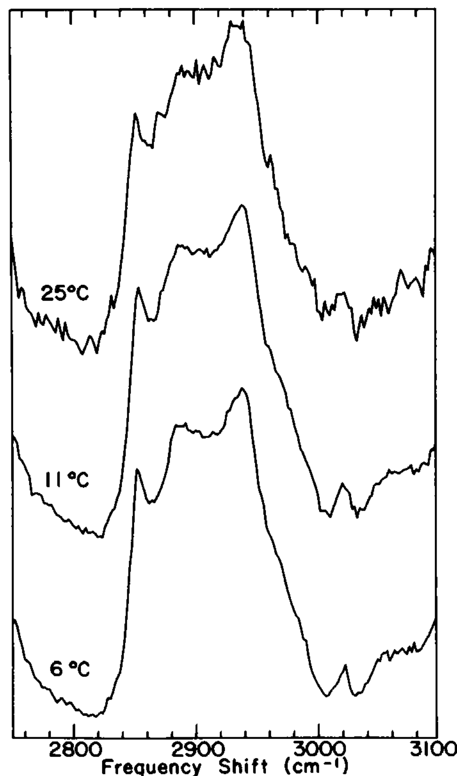

FIGURE 7 The effect of temperature on the C-H stretching region of the Raman spectrum of a garfish olfactory nerve is shown. The conditions are the same as those in Fig. 1.

We are grateful to H. Richard from the Centre de Recherches Paul Pascal and to R. Cavagnat and F. Cruège from the Laboratoire de Spectroscopie Infrarouge for their skillful assistance in obtaining the Raman data. M. Pézolet thanks these laboratories for their hospitality during his sabbatical stay.

This work was supported in part by the Natural Sciences and Engineering Research Council Canada and by the Fonds Formation de Chercheurs et Aide à la Recherche of the Ministère de l'Education of the Province of Québec.

Received for publication 10 April 1984 and in final form 18 September 1984.

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