# Temperature-reversible eruptions of vesicles in model membranes studied by NMR

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ABSTRACT Deuterium ($^2H$) and phosphorus ($^{31}P$) nuclear magnetic resonance (NMR) and freeze-fracture electron microscopy were used to study spontaneous vesiculation in model membranes composed of POPC:POPS with or without cholesterol. The NMR spectra indicated the presence of a central isotropic line, the intensity of which is reversibly and linearly dependent upon temperature in the $L_\alpha$ phase, with no hysteresis when cycling between higher and lower temperatures. Freeze-fracture microscopy showed small, apparently connected vesicles that were only present when the samples were frozen (for freeze-fracture) from an initial temperature of 40–60°C, and absent when the samples are frozen from an initial temperature of 20°C. Analysis of motional narrowing was consistent with the isotropic lines being due to lateral diffusion in (and tumbling of) small vesicles (diameters $\approx$ 50 nm). These results were interpreted in terms of current theories of shape fluctuations in large unilamellar vesicles which predict that small daughter vesicles may spontaneously "erupt" from larger parent vesicles in order to expel the excess area created by thermal expansion of the bilayer surface at constant volume. Assuming that all the increased area due to increasing temperature is associated with the isotropic lines, the NMR results allowed a novel estimate of the coefficient of area expansion $\dot{\alpha}_A$ in multilamellar vesicles (MLVs) which is in good agreement with micromechanical measurements upon giant unilamellar vesicles of similar composition. Experiments performed on unilamellar vesicles, which had been placed upon glass beads, confirmed that $\dot{\alpha}_A$ determined in this way is unchanged compared with the MLV case. Addition of the highly positively charged (extrinsic) myelin basic protein (MBP) to a POPC:POPS system showed that membrane eruptions of the type described here occur in response to the presence of this protein.

## INTRODUCTION

NMR spectra for isotropic liquids are characterized by complete motional averaging of dipolar, quadrupolar, and anisotropic chemical shift (tensor) interactions. For this reason, all structurally/chemically equivalent spins in isotropic liquids give rise to a single central peak that will only be split by orientationally-averaged indirect spin–spin interactions. By contrast, the orientational order of many liquid crystals, such as the $L_\alpha$ phase of phospholipid bilayers, gives rise to non-zero motionally-averaged values of the tensor interactions. Nevertheless, it is not uncommon for NMR spectra of lipid systems in the $L_\alpha$ phase to exhibit a lineshape characteristic of isotropic motions. Such NMR signals may often be attributed to artifacts of the sample preparation such as production of small vesicles or micelles (e.g., by subjecting the sample to freeze-thaw cycling), small background amounts of deuterium ($^2H$) nuclei in the buffer, or chemical degradation of phospholipid molecules resulting in an isotropic motion for some fraction of the sample; alternatively the signal could be due to the presence of cubic phases (Cullis and de Kruijff, 1979; Tilcock, 1986), which give rise to the same type of motional averaging of the tensor interactions as for isotropic liquids. In any case, if the intensity in the isotropic line is small relative to the spectral features being studied, these effects are usually ignored as being insignificant. We recently observed, and studied in detail using NMR and freeze-fracture electron microscopy, an isotropic line from a mixture of POPC (1-palmitoyl-2-oleoyl-phosphatidylcholine), POPS (1-palmitoyl-2-oleoyl-phosphatidylserine), and cholesterol. This line had the unusual feature that its intensity was reversibly and linearly dependent upon temperature: with increased (decreased) temperature the intensity increased (decreased) with no hysteresis apparent when cycling between higher and lower temperatures. Electron microscopy revealed the presence of connected vesicular structures which had some characteristics similar to the vesicle shape fluctuations called "budding" and "vesiculation" (Miao et al., 1991; Seifert et al., 1991; Wortis et al., 1991).

Budding is the name given to the adiabatic and spontaneous "eruption" of a satellite vesicle from a parent vesicle when the satellite remains connected to the parent by a small tube of bilayer membrane. Successive satellite production may subsequently produce bead-like strings of vesicles. Such unusual shapes are also known to occur in red blood cells (e.g., see Deuling and Helfrich, 1977). The shape of a vesicle with fixed surface area and enclosed volume is determined by the minimi-

zation of the total bending energy of the bilayer. Adiabatic vesiculation may occur for a given lipid composition when the satellite vesicles produced have a curvature closer to the spontaneous curvature for those lipids as compared with their curvature in the parent vesicle. Many factors determine whether vesiculation will occur, including temperature, buffer, and membrane composition, especially the presence of charged lipids. The main purpose of this paper is to illustrate the phenomenon and the use of NMR spectroscopy as a method that is complementary to more standard methods used to study membrane mechanical properties (Bloom et al., 1991).

Also, observations will be presented that indicate that such membrane eruptions can occur upon addition of a highly positively charged protein (myelin basic protein [MBP]) to the bilayers studied here.

In model systems the spontaneous production of small vesicles ($\sim$40 nm) has been previously observed for hand vortexed dispersions of myelin lipids (excluding cholesterol) (Fraser et al., 1986), phosphatidic acid, and mixtures of phosphatidylcholine-phosphatidic acid dispersed in water ($<$60 nm) upon adjustment of pH (Gains and Hauser, 1983), and for mixtures of long-chain lecithins (acyl chain lengths $\geq$ 14 carbons) mixed with 20 mol% short chain lecithins (6–8 carbons per acyl chain) (Gabriel and Roberts, 1984). However, none of these cases reported reversibility[1] (in the thermodynamic sense) nor were adequate explanations provided for the phenomena. Other interest in small and large unilamellar vesicles (i.e., SUVs and LUVs) has been aroused by the possibility of using such vesicles to encapsulate drugs for (potentially) their site-specific delivery to target organs (Blume and Cevc, 1990; Madden et al., 1990), and several techniques have been developed for ULV fabrication such as by sonication of multilamellar vesicles (MLVs) (Huang, 1969), the French pressure cell (Milner et al., 1950; Hamilton et al., 1980), extrusion through polycarbonate membranes (Olson et al., 1979; Barenholtz et al., 1979; Hope et al., 1985), and by other methods (Batzri and Korn, 1973; Bruner et al., 1976; Szoka and Paphadjopoulos, 1978; Mimms et al., 1981).

Spontaneous vesiculation in human erythrocytes has also been observed for a variety of triggering mechanisms. Limited examples of these include incubation with sonicated dimyristoylphosphatidylcholine vesicles (Frenkel et al., 1986; Bütikofer et al., 1987), increased intracellular calcium (Allan and Thomas, 1981), ATP depletion (Lutz et al., 1977), and upon addition of a variety of amphiphiles (Hägerstrand and Isomaa, 1989) for exocytotic events, and with, for instance, treatment of ghosts with ATP (Birchmeier et al., 1979) for endovesiculation. In general, the size of the vesicles produced in these experiments seems to be in the range 200 nm–1 μm, which is too large to result in the isotropic lines seen in the experiments described here (below).

In order to understand the origin of an isotropic NMR absorption spectrum for bilayer systems we first discuss a particular example from deuterium ($^2$H) NMR which we will find useful and which, aside from certain specifics of the interaction itself, is representative of other nuclear species as well.

## Some useful theory

In first order perturbation theory, the interaction between the electric quadruple moment of a deuterium nucleus and the (approximately) axially symmetric charge distribution of a carbon-deuterium (CD) bond gives a doublet NMR spectrum having a splitting of $2\omega$ given by

$$\omega = \omega_Q\langle P_2(\cos\Theta)\rangle, \tag{1}$$

where $\Theta$ is the instantaneous, time-dependent angle between the CD bond direction and the external magnetic field, $P_2(x) = (3x^2 - 1)/2$ is the Legendre polynomial of order two, and $\omega_Q/2\pi \approx 125$ kHz is the maximum value of $\omega$ in the absence of any reorientational motion of the CD bond (Davis, 1979). The average ($\langle\ \rangle$) is taken over motions that are "fast on the NMR timescale," i.e., characterized by correlation times $\tau_C$ satisfying $M_2\tau_C^2 = \omega_Q^2\tau_C^2/5 \ll 1$.

For fast conformational and reorientational molecular motions about the local membrane normal $\hat{n}$, which is the local axis of symmetry for the fast molecular motions in fluid membranes, and slow changes of the angle $\beta$ between $\hat{n}$ and the external magnetic field, we obtain

$$\omega = \omega_Q S_{CD} P_2(\cos\beta), \tag{2}$$

where $S_{CD} = 1/2\langle 3\cos^2\theta - 1\rangle$ is the orientational order parameter for the CD bond, and $\theta$ is the instantaneous angle between the CD bond and $\hat{n}$.

The time dependence of $\beta$ is only slow on the NMR timescale for vesicles of large enough radius $R$ that the combined effects of reorientation in a medium of viscosity $\eta$, and molecular diffusion with diffusion constant $D$, expressed in terms of the correlation times $\tau_r$ and $\tau_d$, respectively, satisfy the condition that $M_{2r}\tau_V^2 \gg 1$, where (Bloom et al., 1978):

$$\frac{1}{\tau_V} = \frac{1}{\tau_r} + \frac{1}{\tau_d} = \frac{3kT}{4\pi\eta R^3} + \frac{6D}{R^2}. \tag{3}$$

The effective residual second moment $M_{2r}$ of the motionally averaged NMR spectrum is

$$\begin{aligned} M_{2r} &= \langle\omega^2\rangle_{powder} = (\omega_Q S_{CD})^2\langle[P_2(\cos\beta)]^2\rangle_{powder} \\ &= \tfrac{1}{5}(\omega_Q S_{CD})^2, \end{aligned} \tag{4}$$

and the average is taken over $\beta \in [0, \pi]$ for the powder lineshape (Davis, 1983).

As the vesicle size is decreased, $\tau_V$ decreases rapidly so that eventually the above inequality is violated and one no longer obtains the superposition of doublets normally associated with Eq. 2. For vesicles small enough that the opposite limit, $M_{2r}\tau_V^2 \ll 1$, is satisfied, the quadrupolar splittings are motionally averaged to a single isotropic, Lorentzian line having full width at half-maximum $\delta$ that is related

[1]Addition of myelin basic protein was found to inhibit vesiculation in the case of myelin lipids (Fraser et al., 1986).

to its spin–spin relaxation time $T_2$ by the equation (c.f., Abragam, 1961; Bloom et al., 1978)

$$\pi\delta = \frac{1}{T_2} = M_{2r}\tau_V = \frac{1}{5}(\omega_Q S_{CD})^2\tau_V, \qquad (5)$$

where $\delta$ is the full width at one-half maximum of the experimentally obtained (approximately Lorentzian) lineshape, and $T_2$ is the spin–spin relaxation time (see, for instance, Abragam, 1961; Bloom et al., 1978).

This simple model will be used to show that the isotropic lines noted in our NMR spectra may indeed result from small vesicles of the type we have seen using freeze-fracture electron microscopy.

## EXPERIMENTAL PROCEDURES

## Materials

Perdeuteriated palmitic acid was prepared by standard methods in our laboratory. Avanti Polar Lipids (Birmingham, AL) used this material to produce the single-chain perdeuteriated 1-palmitoyl-2-oleoyl-phosphatidylcholine (POPC-$d_{31}$) used here; they also synthesized the undeuterated POPC and POPS used here. Cholesterol was purchased from Sigma Chemical Co. (St. Louis, MO). Headgroup deuteriated POPC was prepared by condensing POPA and deuteriated choline bromide (MSD Isotopes, Pointe Claire, Quebec, Canada) according to previously described methods (Roux et al., 1983). The nomenclature for the deuteriated choline headgroup is given: $-(PO_4)^-C^\alpha H_2C^\beta H_2N^+(CH_3)_3 \equiv$ PC, where $\alpha$, $\beta$ denote specific carbon atoms in the headgroup.

Spherically supported vesicles (SSVs) consisting of POPC-$d_{31}$:POPS: cholesterol (as above) as single bilayers supported upon spherical glass beads of 0.5 μm size were prepared according to the methods of (Bayerl and Bloom, 1989).

## Sample preparation

The sample preparation procedure was a slightly modified version of published methods (Huschilt et al., 1985). The modification simply involved measuring membrane components using dry weights rather than by titration and, after addition of the buffer (800 μl of 50 mM Hepes, 100 mM NaCl, and pH 7.4, using deuterium depleted water from Sigma Chemical Co.), the sample then underwent four cycles of freezing in liquid nitrogen followed by warming to room temperature (hereafter called freeze-thawing) to equilibrate solute distribution (Mayer et al., 1985). The samples were then centrifuged at 90,000 rpm and 25°C for 1 h, and the pellet was transfered to an NMR tube. All samples composed of POPC:POPS:cholesterol were in the mole ratio 5:1:2.6. The POPC:POPS samples were in the mole ratio 5:1. Myelin basic protein (MBP) was prepared by extraction from isolated bovine myelin using established methods (Lowden et al., 1966) and was stored in the lyophillized form. The amount of POPC-$d_{31}$ used in a sample was typically about 30 mg.

## Freeze-fracture electron microscopy

### Cryofixation and freeze etching

Standard freezing methods were used (Steinbrecht and Zierold, 1987; Robards and Sleytr, 1985; and Plattner and Bachmann, 1982). The sample was raised to a temperature of either 20, 40, or 60°C on a Balzers (Fürstentum, Liechtenstein) gold specimen holder, then frozen in liquid propane at about the temperature of liquid $N_2$. Freeze etching was performed in a Balzers 400 Freeze-etch device. The sample, at a temperature of about 100°C, was cut with a liquid $N_2$-cooled knife. The microtome was maintained at −170°C. The etch time for all preparations was 1 min. The vacuum was maintained at $\sim 10^{-7}$ torr.

### Replication

Platinum/carbon was evaporated using an electronic gun. The shadowing angle was 45°. The film thickness was ~1.5 nm and was measured with a quartz crystal oscillator positioned in the specimen plane. To increase the mechanical stability of the replica, a carbon film of ~10 nm was placed on the sample. The replicas were floated on a 5% solution of sodium dodecyl sulfate.

### Electron microscopy

Replicas were examined in a JEOL 1200 EX scanning transmission microscope (Peabody, MA) in the conventional transmission mode using 80 kV accelerating voltage.

## Nuclear magnetic resonance

$^2$H-NMR experiments were performed on a home-built spectrometer operating at 46 MHz for deuterons (Sternin, 1985). A quadrupolar echo sequence was used: $90_x$-τ-$90_y$-τ-echo, with τ = 60 μs and a repeat time of 150 ms. The 90° pulse length was 4.0 μs. Spectra were recorded with a 2-μs dwell time and at least 50,000 transients collected for signal averaging. Signals were detected in quadrature with phase cycling (Davis, 1979; Rance and Byrd, 1983), and temperature was controlled using a Bruker model BV-T1000 temperature controller (Billerica, MA).

Phosphorus ($^{31}$P) NMR was studied using a Bruker WP-200 spectrometer operating at 81 MHz. Up to 1,000 transients were accumu-

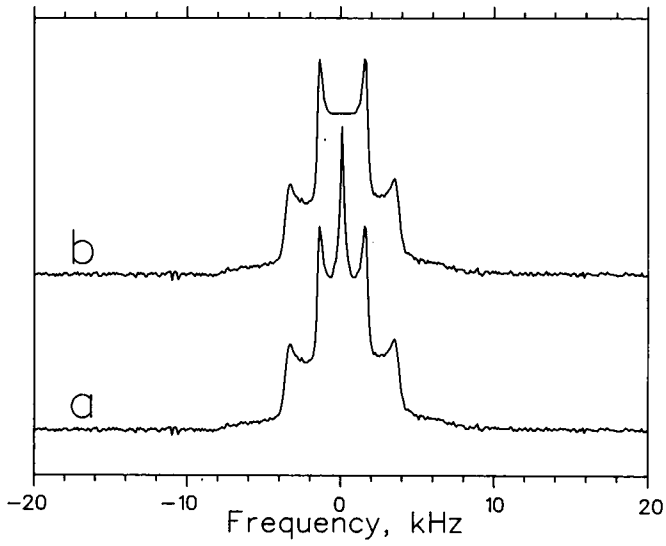



FIGURE 1 $^2$H NMR spectrum of headgroup-deuteriated POPC-$d_4$: POPS:cholesterol (5:1:2.6) m:m:m at 40°C showing the presence of a central isotropic signal (*a*); and the appearance of the same spectrum when the central region has been digitally removed (*b*) in order to determine the fraction of the total spectral intensity which is in the isotropic signal. The isotropic component of the spectrum may be represented as a superposition of two (Lorentzian) NMR signals of equal area, one due to each of the deuteriated carbonyl groups.

lated using a 15-μs 90° pulse length, a 1-s repeat time, and a 20-kHz sweep width. Broad-band proton decoupling was used.

## Determination of isotropic intensity

The method is illustrated here for the case of headgroup deuteriated POPC-$d_4$ in POPC:POPS:cholesterol. In Fig. 1 a spectrum for this sample (lower spectrum) is shown which has an isotropic component. In the upper spectrum the digitized signal for the isotropic part has been removed manually by redigitizing the center points (by eye). The isotropic fraction $f$ is defined as the ratio of the area of the isotropic part removed divided by the area beneath the actual spectrum.

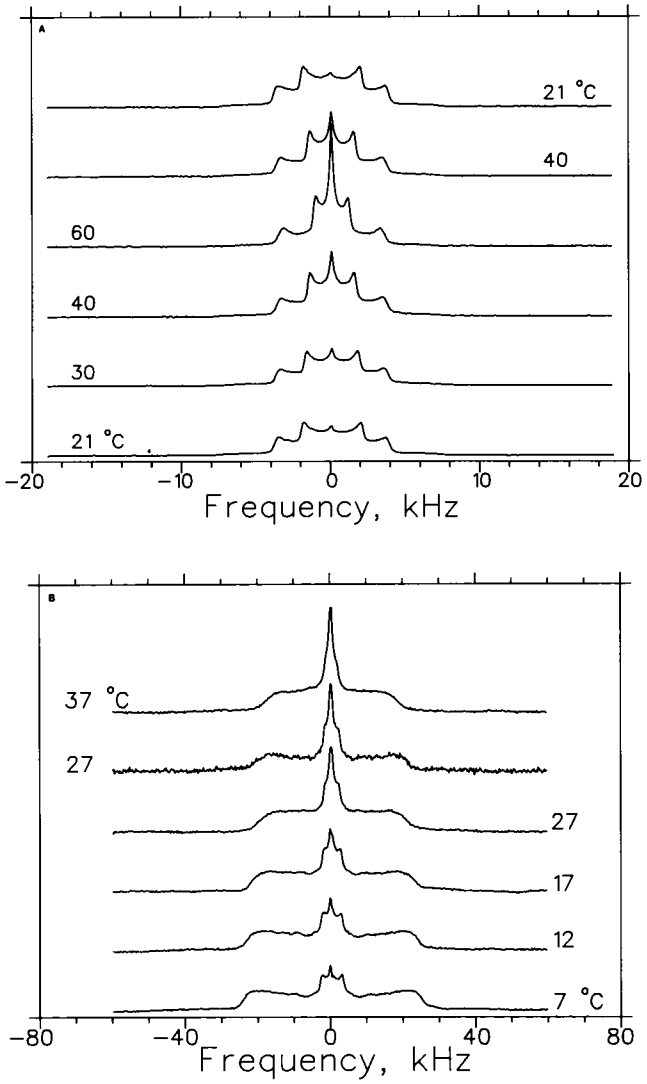
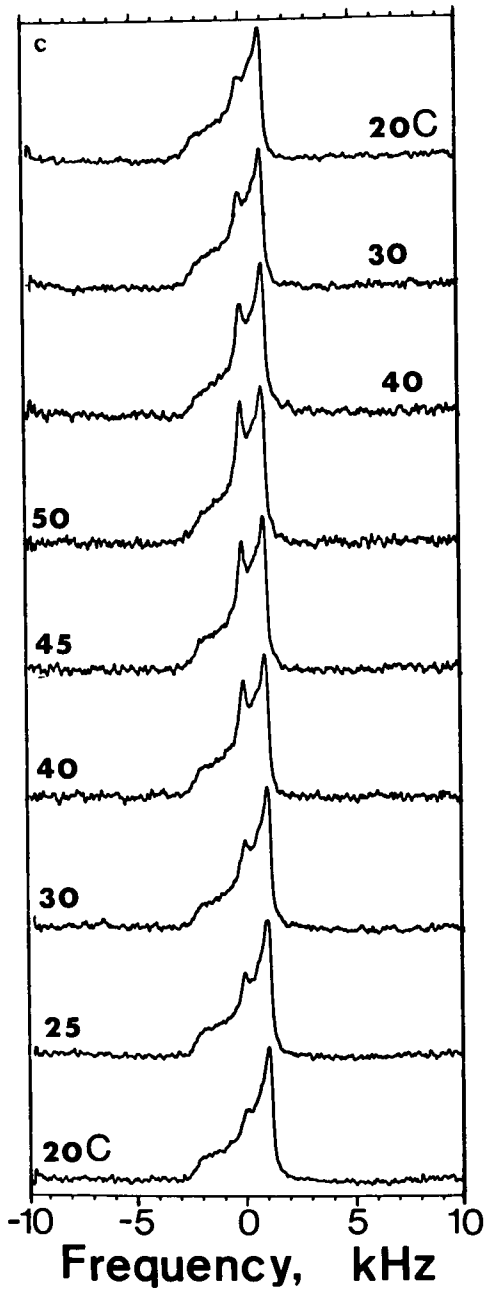



FIGURE 2 Representative spectra obtained at various temperatures are shown for the case of headgroup-deuteriated POPC-$d_4$:POPS: cholesterol by $^2H$ NMR (*a*); for POPC:POPS:cholesterol by $^{31}P$ NMR (*b*); and for chain-deuteriated POPC-$d_{31}$:POPS:cholesterol (*c*). The molar ratio of these membrane constituents for all cases was 5:1:2:6, respectively. The temperature at which each spectrum was recorded is shown on the left side of the figure for increasing temperature between successive spectra and on the right side for decreasing temperature between successive spectra.

## RESULTS

## NMR

Fig. 2, *a*, *b*, *c* shows representative NMR spectra for the cases of headgroup deuteriated POPC-$d_4$ and palmitoyl



chain deuteriated POPC-$d_{31}$ using $^2H$ NMR, and phosphorus $^{31}P$ NMR, respectively, for POPC:POPS:cholesterol mixtures. The temperature at which each spectrum was recorded is noted on the left (right) side of the figure for increasing (decreasing) temperature between consecutive spectra. The primary feature to note is that in each case an isotropic signal appears with increasing (decreasing) intensity for higher (lower) temperatures. The method described above (see Fig. 1) for characterizing the fraction $f$ in the isotropic part of the signal is relatively easily applied for the case of headgroup deuteriated sample (Fig. 2 *a*), and for cases using $^{31}P$ NMR (2 *b*), as compared with the chain deuteriated sample (2 *c*), because there is less error in defining the frequency range over which the isotropic part of the signal is found. The $^{31}P$ NMR spectra are characteristic of lipids in a bilayer structure (Cullis and de Kruijff, 1976, 1978).

These spectra, and similar data for the case of the $^{31}P$ NMR, have been used to determine the isotropic fraction and, in Fig. 3, this is plotted versus the temperature for the various samples. All the data for this figure were obtained by $^{31}P$ NMR except the line labeled b, which was obtained using $^2H$ NMR for the headgroup-deuteriated sample. In all cases there was found to be no

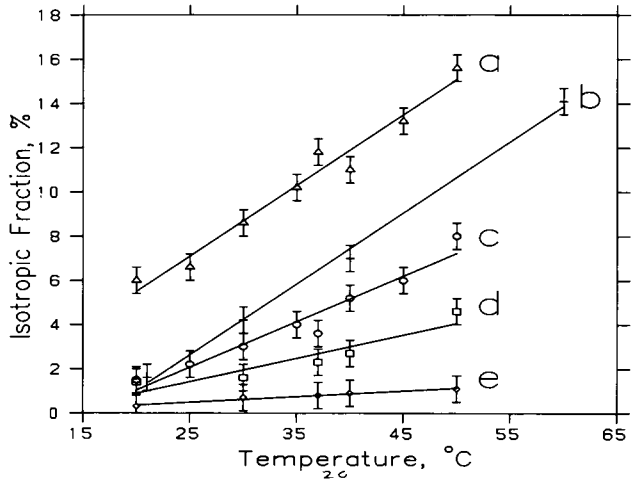



FIGURE 3 The isotropic fraction $f$ of the total spectral intensity contained in the isotropic component of NMR spectra obtained at different temperatures $T$ for various samples of POPC:POPS: cholesterol in the mole ratio 5:1:2.6 (a,b,c), and POPC:POPS in the mole ratio 5:1 (d,e). These samples also differ with respect to their deuteriation and in whether $^2H$ or $^{31}P$ NMR was used to observe them: no deuteriation, by $^{31}P$ NMR (*a*); headgroup-deuteriated POPC-$d_4$, by $^2H$ NMR (*b*); chain-deuteriated POPC-$d_{31}$, by $^{31}P$ NMR (*c*); no deuteriation, by $^{31}P$ NMR (*d*); and chain-deuteriated POPC-$d_{31}$, by $^{31}P$ NMR (*e*). The least-squares fits shown have slopes, in units of $10^{-3}°C^{-1}$, given by 3.2 ± 0.2 (*a*), 3.2 ± 0.1 (*b*), 2.1 ± 0.2 (*c*) 1.1 ± 0.3 (*d*), and 0.26 ± 0.03 (*e*).

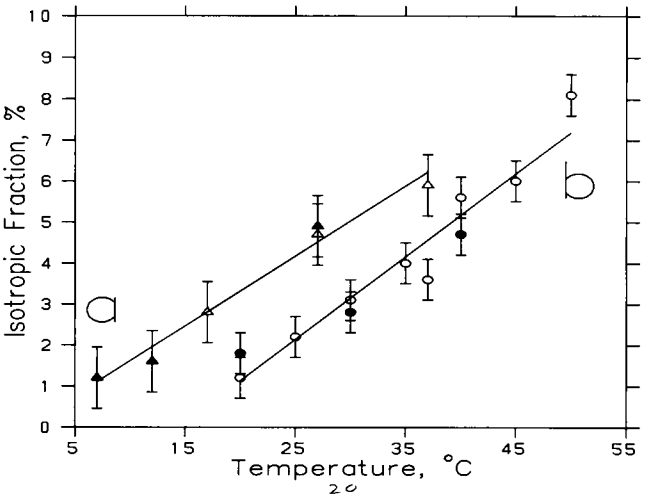
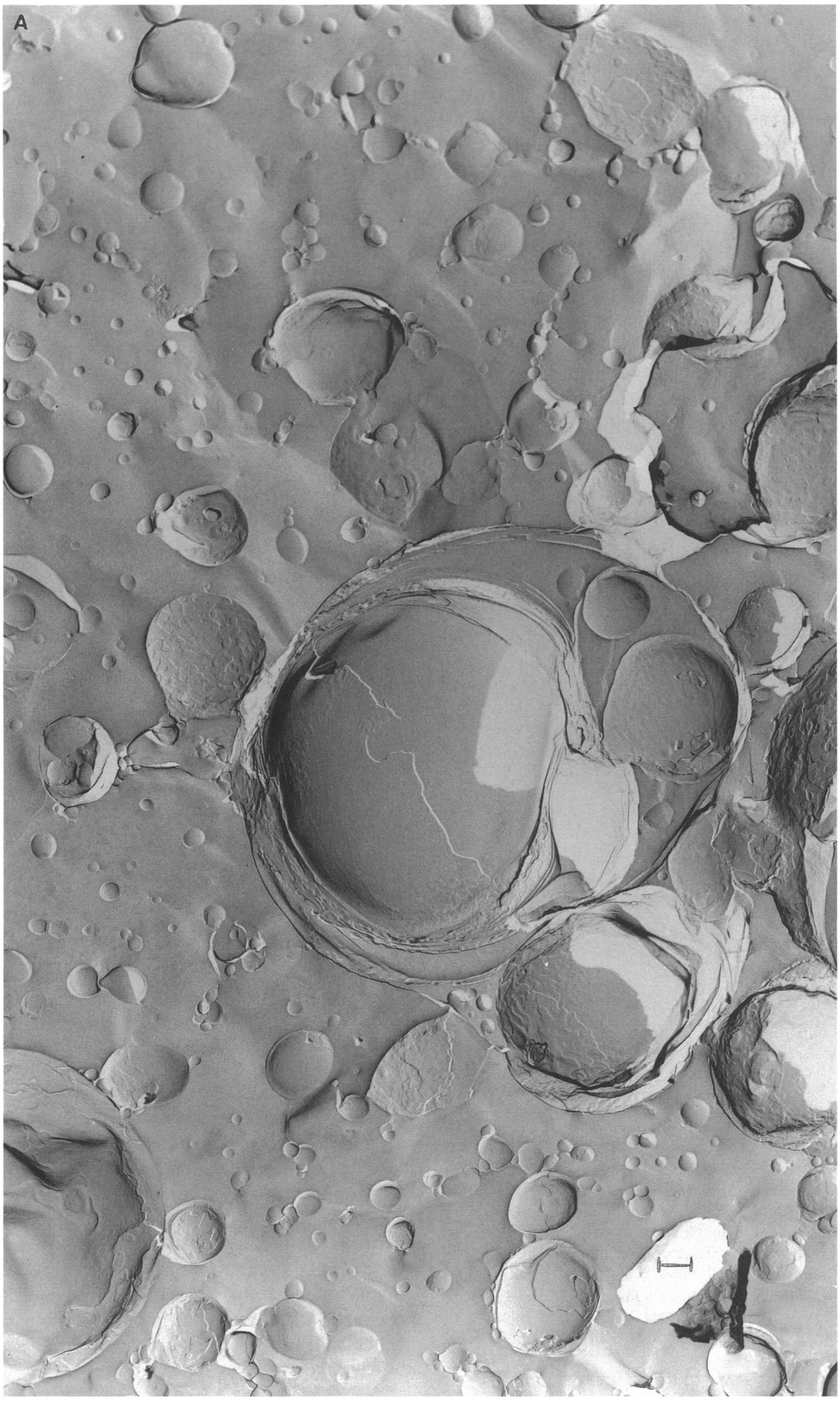



FIGURE 4 The isotropic fraction $f$ is plotted as a function of temperature for chain-deuteriated POPC-$d_{31}$:POPS:cholesterol when in the form of single bilayers formed upon 0.5-μm glass beads (*a*) (by $^2H$ NMR), and in the form of MLVs which have undergone four freeze-thawing cycles (*b*) (by $^{31}P$ NMR). Filled symbols mean decreasing temperature between successive measurements, while open symbols mean increasing temperature.

significant difference between two measurements that were made at the same temperature for a given sample, independent of the recent thermal history in the $L_\alpha$ phase (i.e., no hysteresis). Therefore, for simplicity in Fig. 3, average values have been used when more than one measurement is made at a given temperature. The variation in the isotropic intensity between 20–50°C was in the range of ~1–10%. The first thing to note is that the response of the isotropic fraction to variation in the temperature is linear as shown by the least squares fits (straight lines in Fig. 3). The slopes $df/dT$ are listed in the legend to Fig. 3. Another feature is that, except for sample a, all the samples have similar values for $f$ at the lowest temperature studied, converging to $f$ = 1% at ~20°C. The addition of cholesterol to the POPC:POPS sample, as represented by cases of a, b, and c, is correlated with increased values of the slope $df/dT$ as compared with the cases d and e (no cholesterol). There also appears to be a somewhat peculiar feature: $df/dT$ decreases depending upon whether the palmitoyl chain is perdeuteriated, independent of the presence of cholesterol; e.g., in Fig. 3 compared d with e (no cholesterol), or compare c with either a or b (cholesterol present). This effect is not seen when it is the headgroup that is deuteriated (compare a with b). We believe that this result is likely due to translocation of the palmitoyl and oleoyl chains, known to be up to 20% in the POPC-$d_{31}$ prepared for these experiments (Lafleur et al., 1989), which could alter acyl chain packing in the bilayer.

All the cases represented in Fig. 3 were obtained with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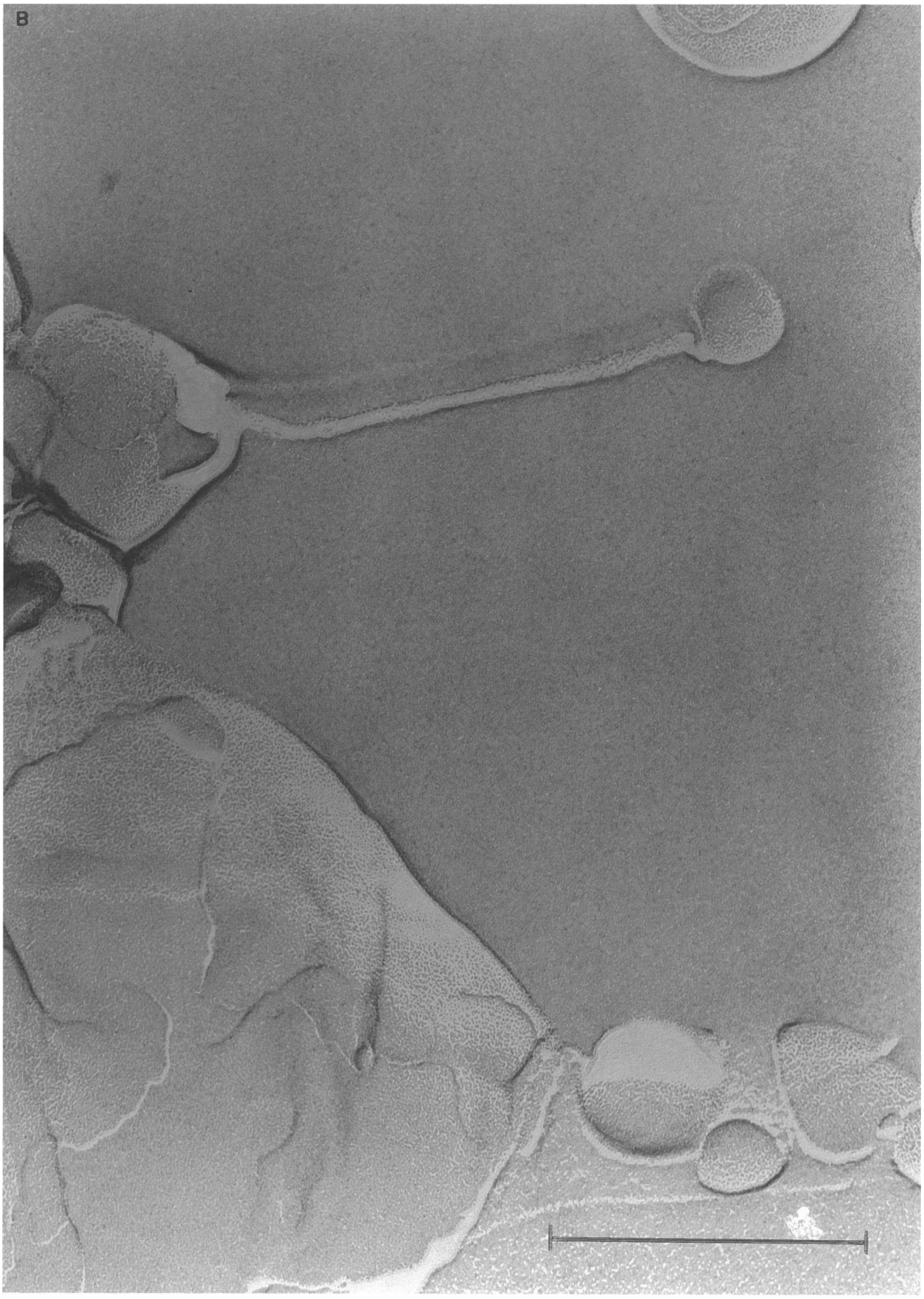
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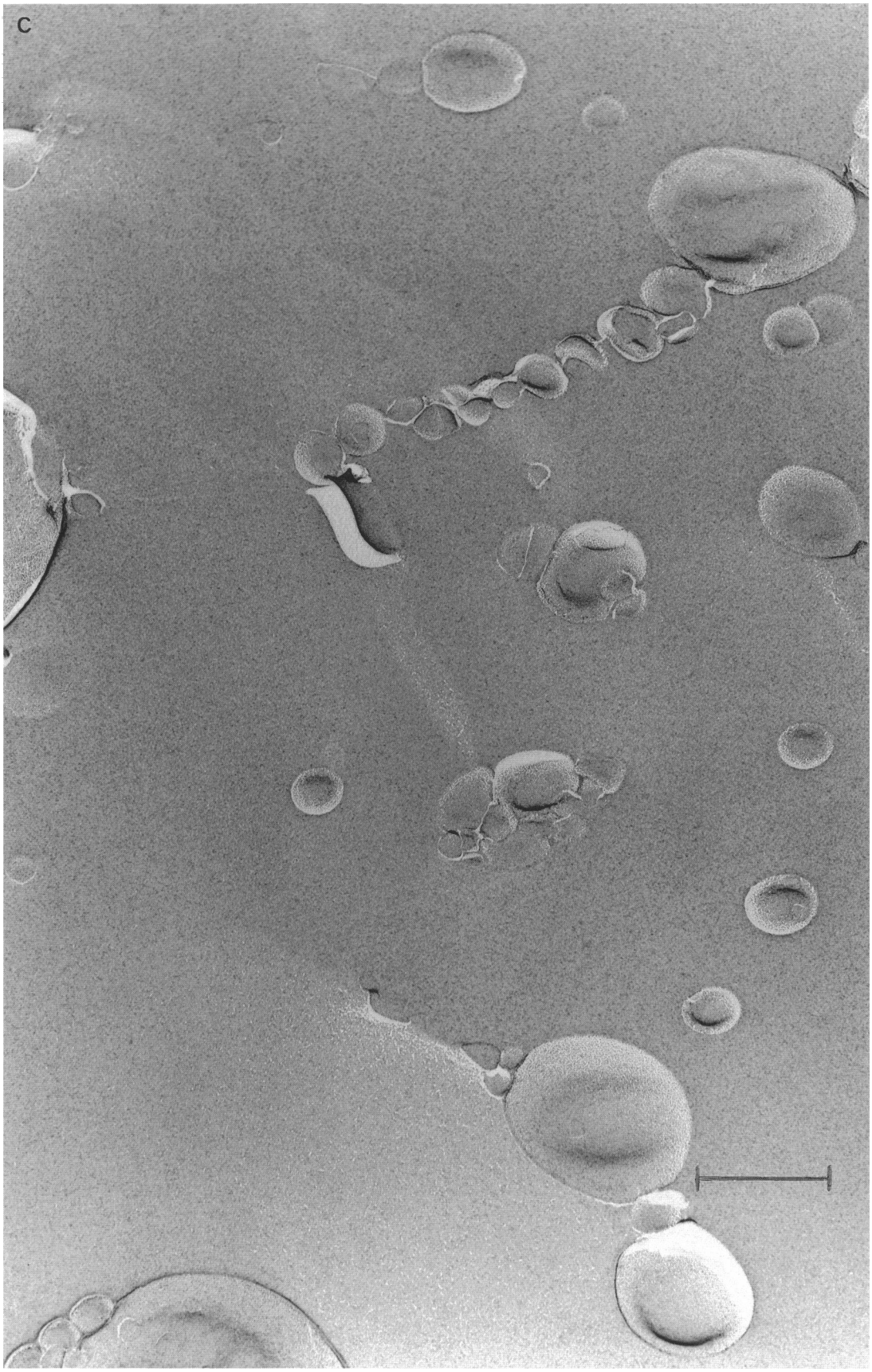

C

MLV samples whose thermal histories included a freeze-thawing stage. Before NMR was performed each sample was removed from a freezer (at $\sim -10°C$) and allowed to equilibrate at room temperature for at least 12 h. Thereafter, the equilibration time at each temperature was $\sim 1$ h before performing the NMR. In order to estimate the importance of the MLV nature of the samples as a contributing factor to the intensity of the isotropic signal, a chain-deuteriated sample containing POPC-$d_{31}$:POPS:cholesterol (in the same proportions as the other samples) was prepared on 0.5 μm glass beads as spherically supported vesicles (SSVs) (Bayerl and Bloom, 1989). It is known that these are unilamellar vesicles of precisely defined spherical shape with an $\sim 1$–1.5-nm thick layer of buffer between the bead and the inner leaflet of a bilayer. Freeze-fracture electron microscopy was not possible on this sample due to the presence of the beads.

In Fig. 4 the isotropic fraction versus temperature is plotted for MLV and the SSV samples that were otherwise identical. Fig. 4 shows that the response is again linear with a slope that is about the same for both samples (i.e., $(2.0 \pm 0.2) \times 10^{-3}°C^{-1}$ for the MLV sample, b in Fig. 4; and $(1.7 \pm 0.2) \times 10^{-3}°C^{-1}$ for the ULV sample); however, the figure indicates that the ULV sample has an isotropic fraction that is increased over that for the MLV sample by a positive constant.

## Freeze-fracture microscopy

In Fig. 5 *a* a typical micrograph for POPC:POPS:cholesterol (5.0:1.0:2.6) is shown; the sample had an initial temperature of 20°C before being frozen in the liquid propane. The fracture faces seen through the bilayers of the MLVs are as expected for samples that have previously undergone freeze-thawing cycles (Mayer et al., 1985) and are not unusual. The size distribution of vesicles ranges from diameters of $\sim 50$ nm to several microns. Also note that small vesicles tend to appear singly and are more or less isolated from the MLV structures. A similar micrograph with no significantly different features was obtained for POPC:POPS samples without cholesterol (data not shown). At higher initial temperatures (40–60°C) the distribution of MLVs appears to be unchanged but a new feature is apparent that is typical of the whole sample: long tube-like structures connected to vesicles are seen to extend from the outer bilayers. Two examples are shown in Fig. 5, *b* and *c*.

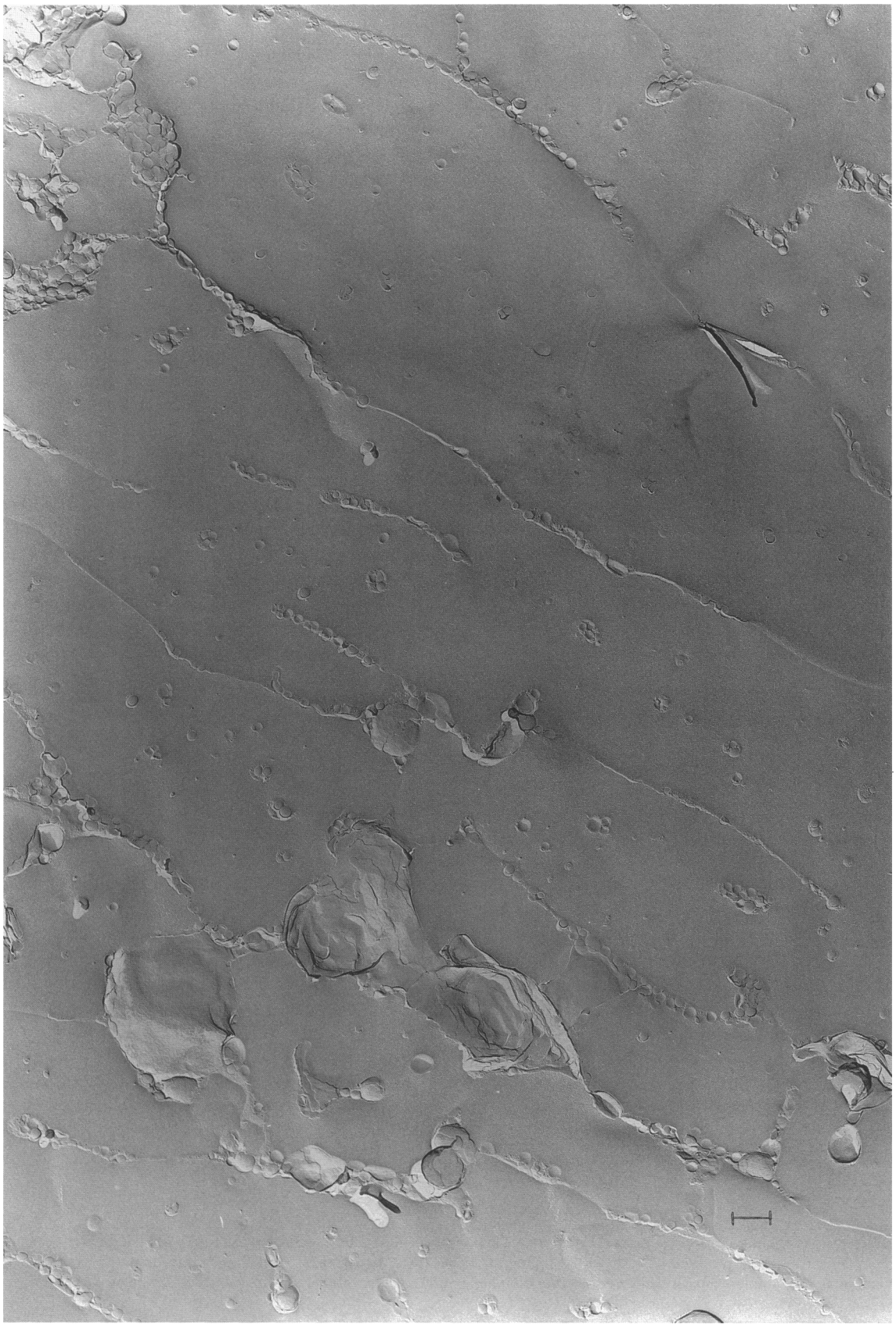
In Fig. 5 *b* is shown a higher power (100k magnification) view of a membrane "eruption" consisting of a vesicle with a diameter of $\sim 50$ nm which is located at the end of a tube of $\sim 0.5$ μm in length. In Fig. 5 *c* a typical series of apparently connected, or at least linearly associated, vesicles is shown. Close inspection of the smaller vesicles shows some fracture surfaces characteristic of MLV structure.

It has been noted by others (Sixl et al., 1984) that a low intensity isotropic line appears in NMR spectra upon addition of $\geq 30$ wt% MBP to a similar system of lipids (DMPC:DMPS excluding cholesterol). We have also observed this phenomenon for the POPC:POPS samples studied here. The freeze-fracture microscopy shown in Fig. 6 for the addition of MBP to a POPC:POPS (5:1) sample again indicates the presence of long bead-like strings of small vesicles; in this case, even when the sample has an initial temperature of only 20°C before being frozen in liquid propane. The vesicles in this case have a tendency to cluster together, as seen in the upper left corner of the micrograph. The same result is found when MBP is added to the POPC:POPS:cholesterol sample of Fig. 5 *a* except that the clumping effect is absent and, although strings of bead-like vesicles are seen, the lengths of these on average appear longer and have fewer vesicles contained within them.

## DISCUSSION

The methods used here to determine the isotropic fraction $f$ are exceedingly simple. While the NMR establishes the presence of isotropic motions, this technique alone is incapable of determining the precise nature of the structures that might be associated with such a motion. However, certain points may still be made on the basis of the NMR data. Firstly, the reversible nature of the isotropic intensity as a function of temperature immediately rules out both the unwanted presence of $D_2O$ in the buffer and/or chemical degradation of phospholipids, as well as ruling out a constant presence of micelles or SUV/LUVs formed in the sample preparation, as possible explanations for the isotropic intensity. Secondly, the $^{31}P$ NMR clearly shows a bilayer signature that argues against any major pres-

FIGURE 5 Micrographs obtained by freeze-fracture microscopy for samples of POPC:POPS:cholesterol. Micrograph a shows typical MLVs when the sample was frozen in liquid propane from an initial temperature of 20°C. Micrographs b and c show higher power views of some typical structures common to the samples which were frozen starting from higher initial temperatures: 60°C (*b*), and 40°C (*c*). The bars in the pictures each represent 200 nm.



ence of hexagonal ($H_{II}$) phase lipid organization (Cullis and de Kruijff, 1976, 1978). Also, PS and PC lipids are known to be bilayer stabilizing and they tend not to form nonbilayer phases (Tilcock, 1986). It is conceivable that cholesterol could incline the system towards such structures but the freeze-fracture microscopy argues against this interpretation because there is no evidence of cubic phases being present, nor is there evidence of inverted micelles. The only significantly different feature in the micrographs, which is temperature dependent, is the presence of the apparently connected regions of small vesicles described above.

It is well known that cooling rates in lipid systems of the type studied here, using a liquid propane bath kept at approximately the temperature of liquid nitrogen, are on the order of $\sim -10^3$°C/s (Plattner and Bachmann, 1982). Therefore, the cooling time required in our experiments for the sample to go from the fluid to the gel phase is expected to be between 20–60 ms. Since eruptions in giant unilamellar vesicles have typically been observed to have durations on the order of seconds and longer (personal observations of eruptions seen on video recordings made by E. Evans), it is, therefore, not surprising that the electron microscopy is able to detect the presence of vesicles that have initially erupted at the higher temperature and then have been, subsequently, "frozen in."

The high resolution of the spectrum for the headgroup deuteriated sample allows a further check. Close inspection of the spectrum given in Fig. 1 suggests that the isotropic line seen there is actually a superposition of two Lorentzian lineshapes, as would be expected for motional narrowing of the two pairs of spectroscopically distinct deuterons at the α and β positions of the choline headgroup. The predicted width, according to the model suggested in the introduction, may be determined. Noting that the characteristic vesicle size produced has a radius on the order of $R \approx 25$ nm then, using Eq. 3 with $\eta = 10^{-2}$ Poise as the viscosity of water, $D = 5 \times 10^{-8}$ cm$^2$/s as the diffusion constant (Bloom et al., 1991), and a temperature $T \approx 50$°C, we find $\tau_r \approx 40$ μs and $\tau_d \approx 20$ μs, which gives $\tau_V \approx 13$ μs. Also, Eq. 4 may be used to determine $M_{2r}$ for the α and β carbon positions to obtain $M_{2r}^{\alpha} = 3.7 \times 10^8 s^{-2}$ and $M_{2r}^{\beta} = 6.2 \times 10^7 s^{-2}$. These values for $\tau_V$, $M_{2r}^{\alpha}$, and $M_{2r}^{\beta}$ satisfy $M_{2r}\tau_V^2 \ll 1$. Using Eq. 5 we can now predict the full width at one half maximum, δ, of the Lorentzian lineshapes which would result from isotropic motional averaging of the α and β quadrupolar splittings and find: $\delta_\alpha = M_{2r}^{\alpha}\tau_V/\pi = 1.5$ kHz and $\delta_\beta = 260$ Hz. These values agree favorably with the approximate widths of 1.6 kHz and 380 Hz for the α and β positions, respectively, as measured directly off the isotropic peak in Fig. 1, suggesting that the isotropic NMR lines are consistent with the vesicles seen using electron microscopy.

In these experiments lipids move between anisotropic and isotropic structures reversibly with temperature. If the simplifying assumption is made that all the extra membrane area induced by increasing the temperature goes into the isotropic structures, and if the area per lipid is approximately the same in the lamellar and vesicular regions, then it is easy to show that the coefficient of areal expansion $\dot{\alpha}_A$ (Bloom et al., 1991) is related to the intensity of the central peak by

$$\dot{\alpha}_A = \frac{1}{A}\frac{dA}{dT} \approx \frac{df}{dT}. \quad (6)$$

The data of Fig. 2 for the POPC:POPS:cholesterol samples gives $df/dT \approx (2\text{–}3) \times 10^{-3}$°C$^{-1}$, which is remarkably close to the value of $\dot{\alpha}_A \approx 2 \times 10^{-3}$°C$^{-1}$ obtained by (Needham et al., 1988) for mixtures of DMPC and cholesterol. In any case, it is known that the availability of excess area for large scale thermal undulations in LUVs can result in an unstable vesicle surface which has a tendency to spontaneously erupt into satellite vesicles connected by microscopic umbilical tubes (Evans and Rawicz, 1990). It should be kept in mind that the lipid mixtures being compared have different molecular compositions and different mechanical forms: multilamellar vesicles (MLVs) in our measurements and giant unilamellar vesicles (GUVs) in the micromechanical measurements (Needham et al., 1988). Nevertheless, we believe that the order of magnitude agreement between $df/dT$ and $\dot{\alpha}_A$ is significant and indicates that the constraints imposed by the stacked lamellae of the MLVs do not significantly limit this vesicular-type of area expansion. Unfortunately in this case, the process of vesiculation is generally described for LUVs and we know of no theoretical description of budding or vesiculation occuring in MLVs (where steric considerations may be important).

The least squares fits in Fig. 3 show that the addition of 30% cholesterol to bilayers composed of POPS:POPC increase $df/dT$ by $\sim 2 \times 10^{-3}$°C$^{-1}$ for both the chain-deuteriated POPC, from $(0.26 \pm 0.03) \times 10^{-4}$°C$^{-1}$ to $(2.1 \pm 0.2) \times 10^{-3}$°C$^{-1}$, and for the non-deuteriated POPC, from $(1.1 \pm 0.3) \times 10^{-3}$°C$^{-1}$ to $(3.2 \pm 0.2) \times$

FIGURE 6 Freeze-fracture micrograph (for freezing from an initial temperature of 20°C) for a sample composed of POPC:POPS in the molar ratio 5:1 which has had ~50 wt% myelin basic protein (MBP) added to it. The bead-like strings of small vesicles characteristic of this sample are absent in corresponding micrographs for similar samples that lacked MBP (not shown but similar to Fig. 5 *a*). The bar represents 200 nm.

$10^{-3}°C^{-1}$. This suggests that the eruptions occur more readily in the presence of cholesterol than in its absence. This result is not necessarily expected on the basis that the increased bending stiffness associated with cholesterol addition might be expected to inhibit the process of vesiculation by making regions of higher curvature less accessible in terms of energetics. However, it may be that cholesterol somehow lowers the energy barrier to vesiculation, perhaps due to its ability to rapidly "flip-flop" between the two leaflets of the bilayer. This could allow for local fluctuations of increased curvature so providing a focus for the eruption event.

Another question concerns whether the lipid composition is identical in both the parent and the daughter vesicles. For instance, considerations of lipid polymorphism would preferentially locate the (charged) POPS in vesicles of higher curvature compared with POPC. The presence of MBP may also influence the lipid distribution. In the case considered in Fig. 6 the vesiculation may be due to MBPs expected tendency to associate preferentially with negatively charged lipids, such as POPS (Boggs et al., 1977; Smith and McDonald, 1979), possibly resulting in perturbations from ideal mixing of the lipids present and, therefore, permitting a localized variation of the bulk lipid composition which could promote vesiculation. However, charge of the lipids is expected to be only one of many factors (buffer, pH, etc.) that need to be studied in association with vesiculation effects.

Fig. 3 suggests that $df/dT \approx (2.0 \pm 0.2) \times 10^{-3}°C^{-1}$ in the POPC-$d_{31}$:POPS:cholesterol samples, independent of whether these are MLVs or LUVs on glass beads. However, there is still a constant difference in $f$ between these two samples that needs to be explained. Unfortunately, the difference in the thermal histories of these two samples (the MLV sample underwent four freeze-thawing cycles in the initial sample preparation) does not permit definitive comments to be made regarding the apparent relative increase in the absolute intensity of the isotropic peak for the LUV sample; it may be that stresses associated with the freeze-thawing procedure do not relax on the time scale of $\sim 12$ h which was used as an equilibration time in these experiments.

## CONCLUDING REMARKS

The work described here shows that some lipids in the MLV systems studied undergo a temperature-dependent and reversible change in the degree of motional averaging of their NMR spectra, consistent with spontaneous vesiculation (or the MLV analogue of this) occurring in response to changes in temperature. The novel method illustrated here for estimating the coefficient of area expansion $\dot{\alpha}_A$ from NMR measurements gives results in agreement with existing micromechanical measurements for $\dot{\alpha}_A$ (Needham et al., 1988) in similar systems.

Although we have chosen to illustrate these effects with a rather specialized ternary lipid system, it should be made clear that we believe this phenomenon to be common to many lipid systems and has probably been observed in passing, but not interpreted, by spectroscopists before now. Much work is presently being done to clarify the physics underlying three-dimensional membrane conformations (Lipowsky, 1991; Wortis et al., 1991; Seifert et al., 1991; Miao et al., 1991; Evans and Rawicz, 1990; Mutz and Helfrich, 1989; and Sackman et al., 1986). The significance of spontaneous eruptions of the type described here remains to be demonstrated for the larger community interested in membranes (especially the biological and medical side).

In this regard, the addition of the highly positively charged MBP to the POPC:POPS system has been included to illustrate the point that membrane eruptions/vesiculations can occur in response to the presence of a protein. The potential biological importance of proteins influencing the eruption process should not be underestimated: the possibility of cellular (genetically determined) control of eruptions, although in present experimental terms is highly speculative, is a worthwhile consideration even at the outset of emerging experimental programs to study these events. For example, the local initiation of surface eruptions in bilayer subsystems within the cell could contribute to our understanding of such things as cell–cell contacts and transport of cellular materials.

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