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A SPIN LABEL METHOD FOR MEASURING INTERNAL VOLUMES IN LIPOSOMES OR CELLS, APPLIED TO Ca-DEPENDENT FUSION OF NEGATIVELY CHARGED VESICLES

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A new spin label – broadening agent system for measuring trapped volumes of vesicles or cells is described. The method seems to be more advantageous than existing procedures when volumes of highly negatively charged vesicles are to be determined. The membrane permeable spin label is TEMPONE (2,2,6,6-tetramethyl piperidone-*N*-oxyl), and the nonpermeable broadening agent is chromium oxalate ($K_3Cr(C_2O_4)_3$). Absolute values for the trapped volumes down to 0.1% in 0.1 ml can be measured with an accuracy of about ±(1–10%). The method is used to study the final volume of fused phosphatidylserine vesicles as a function of the temperature at which the Ca-induced fusion takes place.

Introduction

When investigating various properties of cells or artificial vesicles, the internal aqueous volume is often an important parameter. Cell volumes can be measured by a variety of methods, including Coulter Counting, dynamic and conventional light scattering, or labeling by radioactive, fluorescent, biochemical or paramagnetic molecules.

The paramagnetic methods have especially been developed by Keith, Snipes and coworkers, and have been used for measuring internal volumes and viscosities in cells [1,2]. Signals from external and internal spin label molecules were distinguished by using $NiCl_2$ or $K_3Fe(CN)_6$ in solution, so that the external signal was broadened (and reduced) by exchange and dipole-dipole interactions.

Berg and Nesbitt [3] introduced the combination of TEMPAMINE and chromium oxalate ($K_3Cr(C_2O_4)_3$) which proved to be very useful in exploring the interior of thylakoids. However, for highly anionic small unilamellar vesicles no reported paramagnetic method can be used for volume measurement because they violate at least one of the following criteria: (1) Neither the spin label nor the broadening agent can bind to the negatively charged phospholipids. (2) The sensitivity, mainly determined by the degree of broadening of the external signal, has to exceed some practical threshold.

In the present paper we introduce a combination of the spin label TEMPONE and the broadening agent chromium oxalate. This combination fulfills the above criteria. Furthermore, since TEMPONE is neutral, the results will not be affected by pH differences or electrostatic potentials across the membrane. However, since TEMPONE also goes into the lipid bilayer, the problem with the present method is to separate the TEMPONE signal from the internal aqueous space from those due to spin labels in the lipid or external aqeous space. By using the high-field ESR line this can actually be done by a subtraction process. This process is not exact, but the uncertainty introduced is

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Abbreviations: TEMPONE, 2,2,6,6-tetramethyl-4-oxopiperidine-1-oxyl; TEMPO, 2,2,6,6-tetramethylpiperidine-1-oxyl; TEMPOL, 4-hydroxy-2,2,6,6-tetramethylpiperidine-1-oxyl; TEMPAMINE, 4-amino-2,2,6,6-tetramethylpiperidine-1-oxyl; Pipes, piperazine-*N*,*N*'-bis(2-ethanesulphonic acid).

negligible in most cases. The method has been used to measure trapped volumes in vesicles after Ca^{2+}-induced fusion of small unilamellar vesicles made from phosphatidylserine. The dependence of final volume on the temperature of incubation with Ca^{2+} shows interesting characteristics.

Methods

Phosphatidylserine (bovine brain) and L-α-phosphatidylcholine (egg) were purchased from Avanti Biochemicals Inc. (Birmingham, AL). The compounds were >99% pure as checked by TLC. Cholesterol was purchased from General Biochemicals and was >99% pure. Potassium trioxaltochromiate was purchased from ICN Pharmaceuticals (Plainview, NJ). D-[1-^{14}C]-Glucose was derived from The Radiochemical Centre, Amersham, and the activity was 3 mCi/mmol. TEMPONE (2,2,6,6-tetramethylpiperidone-*N*-oxyl) was synthesized according to standard procedure [4].

Small unilamellar vesicles were made by sonication on a L&R Ultrasonic bath type sonifier (type UC-3L) until clear (140 W, 0°C, 2 h typically). The suspension was centrifuged at 30000 rev./min for 10 min to remove large vesicles or lipid aggregates. Most of the vesicles were unilamellar with a diameter of 250–500 Å as judged by negative staining electron microscopy.

Large vesicles were made by reverse phase evaporation according to Szoka et al. [5] with small modifications. The lipid composition was egg phosphatidylcholine : phosphatidylserine : cholesterol = 10 : 1 : 10 (mol ratio), with 21 μmol total lipid suspended in 2 ml aqueous phase. In the experiments where radioactivity was used this phase contained 4 mM unlabelled glucose and 1 μCi/ml labelled glucose in the original preparation. Untrapped labelled glucose was washed away by dialysis or centrifugation.

The buffer consisted of 160 mM KCl and 40 mM Pipes adjusted to pH 6.8 by KOH. The chromium oxalate stock solution (made fresh every two weeks) was 150 mM, pH adjusted to pH 6.8 by KOH when necessary. The chromium oxalate purity was given as 95% by the manufacturer, and was checked spectroscopically [6] by us with the same result (GCA/McPherson Instrument EU-700 series). The osmolarity for the buffer and chromium oxalate solutions were both roughly 380 mosM measured on a Osmette 2007 precision osmometer. It should be noted that the ratio of osmolarity to molarity for chromium oxalate is slightly concentration dependent but roughly 2.85, 2.65 and 2.50 at the concentrations ≤50, 100 and 200 mM, respectively. All solutions used were obtained by mixing buffer, chromium oxalate, 400 mM glucose and 200 mM TEMPONE stock solutions in proper proportions.

The Pressman cell [7] equilibration experiments were carried out with 10 ml 3.6 mM TEMPONE in CCl_4 in the bottom phase, 4 ml buffer and 4 ml 60 mM chromium oxalate in buffer in the two upper phases. The bottom phase was stirred by a magnetic stirrer at room temperature. The equilibration of label in the two uppe phases was monitored by taking 200-μl samples. Typically, 4–6 h was needed for equilibration in our set-up, but samples used in the calculations were taken after about 12 h incubation. The final TEMPONE concentrations in the aqueous phases were about 0.8 mM. Equal volumes of sample from the Pressman cell and from the opposite kind of aqueous solution were mixed, and the ESR spectra recorded. In the final solutions the chromium oxalate concentration was the same (30 mM) as verified by linewidth measurements.

The radioactivity was measured on a Beckman LS-230 Liquid Scintillation System at typically 10^4 counts/min, 20 min counting.

The ESR measurements were carried out at X-band with a Varian E9 ESR spectrometer equipped with dual rectangular cavity. A standard flat cell was used with a strong pitch as reference. The detection mode throughout this work was 100 kHz first harmonic absorption.

Results

Fig. 1 demonstrates the dramatic effect of chromium oxalate on the ESR spectrum from 1 mM TEMPONE in solution. Due to exchange interaction and dipole-dipole interactions the usually narrow lines from TEMPONE are broadened heavily by chromium oxalate. When the linewidth (Γ) increases, the amplitude (A) of the lines decreases according to the well known relationship:

$$\Gamma^2 \cdot A = \text{constant}$$

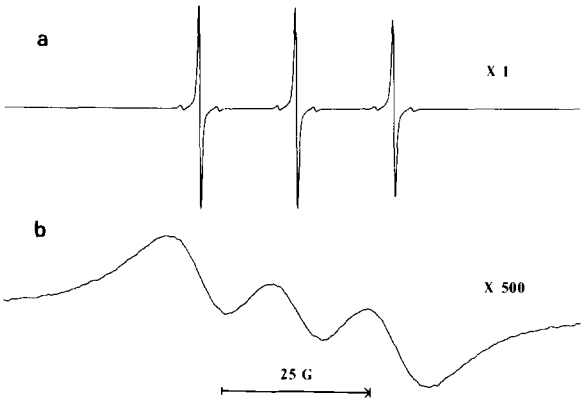

Fig. 1. ESR spectra from 1 mM TEMPONE in absence (a) and in presence of 60 mM chromium oxalate (b). The buffer is described in Methods. The microwave power was 2.2 mW and the modulation amplitude was 0.16 and 1.6 G in (a) and (b), respectively. The indicated amplification factor corresponds to a case where the modulation amplitude was equal in both records.

This is the reason for the dramatic drop in amplitude for the two spectra in Fig. 1.

When the chromium oxalate concentration is varied and the amplitude of the high-field line in the spectrum is measured, one gets the relationship seen in Fig. 2. At 60 mM chromium oxalate the amplitude of the high-field line is only 0.1% of that observed in absence of chromium oxalate, but this depends on the spin label concentration, on the power saturation, and on the modulation amplitude. The deviation in Fig. 2 from a straight line is mainly due to overlap between the different ESR lines at high chromium oxalate concentration.

In a volume measurement the vesicle or cell suspension is first mixed with chromium oxalate and spin label solutions. The spin label penetrates the membrane very rapidly so that full equilibration often is complete within 1 min. However, the chromium oxalate leaks only very slowly through the membrane, so that in accordance with the result of Berg and Nesbitt [3], we find no measurable leakage of chromium oxalate into our vesicle systems during the first few hours after mixing. (Typically 10–12 h after mixing is needed before any increase in the linewidth is detected). Therefore, no broadening of the spectrum from trapped spin labels should be observed during the measurements, while the spectrum from spin labels in the external aqueous space is broadened

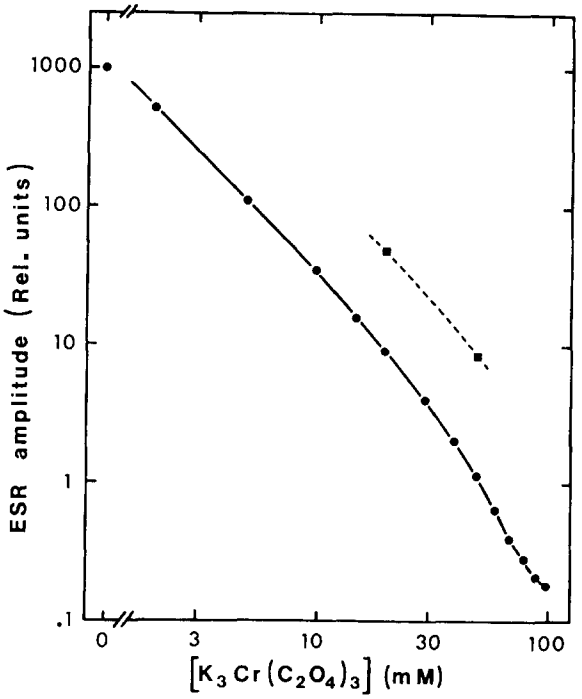

Fig. 2. Relative ESR amplitude of the high-field TEMPONE line as a function of chromium oxalate concentration (●———●). The TEMPONE concentration was 0.1 mM, and the correct chromium oxalate concentrations were obtained by mixtures of the two solutions: 110 mM KCl, 40 mM Pipes (pH 6.8) and 100 mM $K_3Cr(C_2O_4)_3$ (or 75 mM $K_3Fe(CN)_6$). Modulation amplitude and microwave power were 0.16 G and 1.0 mW, respectively, for low chromium oxalate concentration but were increased under control to get better signal-to-noise ratios at high chromium oxalate concentrations. For comparison, the reduction in amplitude when $K_3Fe(CN)_6$ was used instead of chromium oxalate is indicated by (■———■).

extensively. The resulting spectrum (high field line) may, in unfavourable experiments, appear like that given in Fig. 3. Three components can be distinguished: a resonance due to spin labels in the external aqueous space (marked 1), in the lipid phase (marked 2), and in the internal aqueous space (marked 3). Component 2 is shifted toward lower fields in accordance with known effects [8]. The separation of the components can be carried out according to a procedure given in the figure legend. It is difficult to arrive at a correct signal for the spin labels in the lipid phase since the resonance from this phase changes when we put chromium oxalate inside the vesicles. This change is both in amplitude and in

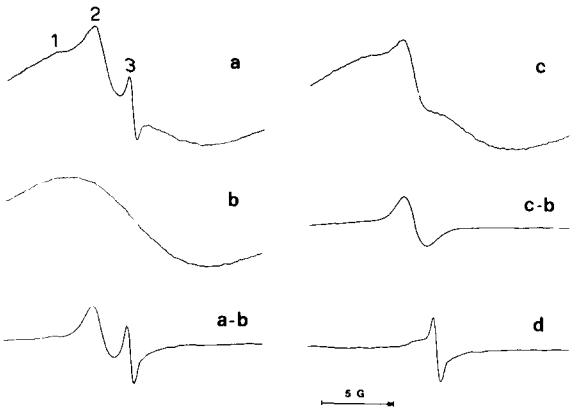

Fig. 3. The high-field lines in the ESR spectrum from the following solutions: Small unilamellar vesicles made from phosphatidylserine (originally 5 mg/ml): chromium oxalate solution = 2 : 1 (a), buffer: chromium oxalate solution = 2 : 1 (b), and the same as in a but freeze thawed five times (c). In all the samples TEMPONE was added to give a final concentration 1 mM. The spectra a–b and c–b are obtained by subtracting (by hand) 100% of b from a and c, respectively. Spectrum d is obtained by subtracting 121% c–b, shifted 0.35 G toward lower fields, from a–b. It should be noted that the peak to peak linewidth in d was within the experimental error the same as with 1 mM TEMPONE in buffer, thus indicating no apparent increase in internal viscosity in these systems. Further discussion in the text.

resonance frequency. (This is the reason for the need of strange factors as 121% and 0.35 G in the procedure to obtain spectrum d in Fig. 3.) It is therefore not possible to determine with certainty if the shoulders on the line d in Fig. 3 are artifacts due to incorrect subtraction or if they really are an integral part of the resonance from the spin label in the internal aqueous space. In this case where the trapped volume in the vesicle suspension is only 0.15%, and the partitioning into the lipid is very high (small unilamellar vesicles of bovine brain phosphatidyl serine), the uncertainty in the amplitude of the narrow signal is roughly ±10%. In most cases the uncertainty is much less, typically 1–2%, and the amplitude can be estimated directly from the spectrum without lengthy subtraction procedures.

From the amplitude of the narrow component like the one marked d in Fig. 3, and the amplitude of a standard sample consisting of TEMPONE in the same concentration (no chromium oxalate), the absolute value of the trapped volume is obtained. In reasonable agreement with other determinations [9], we estimate the trapped volume of the small unilamellar vesicles to be 0.3 l/mol phosphatidylserine if 100% recovery of the lipid is assumed.

In the procedure mentioned above for measuring volumes on an absolute scale it was implicitly assumed that the ratio of the local concentration of spin labels inside the liposomes or cells to the concentration outside in the presence of chromium oxalate was equal to one. However, the broadening of TEMPONE ESR lines by chromium oxalate is so efficient that it might suggest a kind of specific interaction between the two molecules. If that is the case, the ratio mentioned above might, in the present experiments, be different from one. This problem was elucidated using a Pressmann cell [7] as described above. The measured ESR amplitudes yielded the following result:

$$[\text{TEMPONE}]_{\text{chromium oxalate}}/[\text{TEMPONE}]_{\text{buffer}} = 0.99 \pm 0.01$$

This finding indicates that the TEMPONE concentration inside the vesicles or cells will, in fact be equal to that outside. In contrast to charged spin labels, this holds true even if there is a gradient of pH or electrostatic potential across the membrane.

The spin label concentration inside the lipid can be estimated from spectra like the one marked c–b in Fig. 3. In this experiment roughly 2% of the spin labels are in the lipid phase. For precise measurements, this contribution must be corrected for. However, in most cases it introduces a smaller uncertainty than the amplitude measurements. Furthermore, if the reference sample (no chromium oxalate) contains the same lipids as the sample of interest, the error is largely reduced. The reason for this is as follows: the same percentage of the spin label molecules, in both the reference sample and the sample of interest, will partition into the lipid phase. As a result, the signal from the spin labels in the aqueous phase will be reduced almost proportionally with the percentage spin labels in the lipid phase. This is true because of differences in linewidths and the small degree of overlap between lines 2 and 3 in Fig. 3. Thus, the amplitude of the reference signal (corresponding to line 3 in Fig. 3) is much closer to a '100% trapped volume'

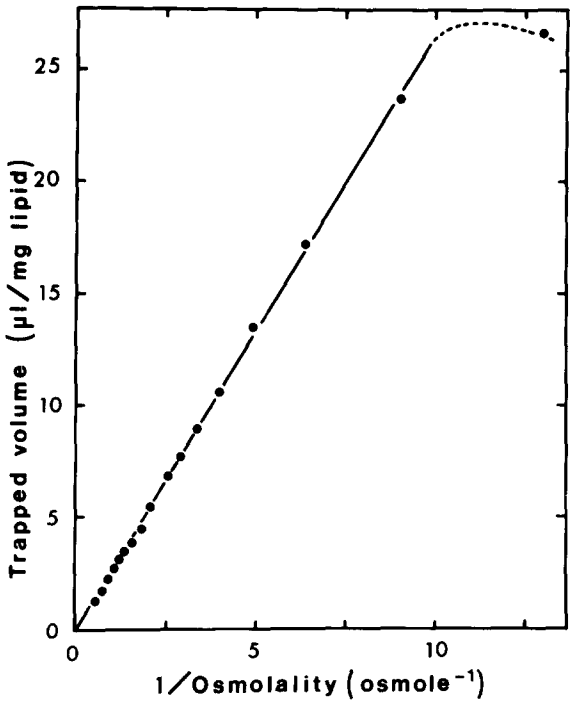

Fig. 4. Trapped volume of reverse phase evaporation vesicles as a function of the osmolality in the external medium. The vesicles were made at about 100 mosmol (40 mM KCl, 10 mM Pipes (pH 6.8)). The final samples contained 10 mM Pipes, 20, 40 or 60 mM chromium oxalate, 1 mM TEMPONE, and various KCl concentrations to obtain the different osmolalities. The pH of all samples was 6.8. All osmolalities were measured on an osmometer.

signal, as it is meant to be, if lipids are included in the reference sample.

To demonstrate how the volume measurement method can be used, we investigated the variation in trapped volume of reverse phase evaporation vesicles [5] as a function of osmolality. To carry out a pretty large variation in osmolality, we had to use intervals with different chromium oxalate concentration (20, 40, and 60 mM). The results are given in Fig. 4 and are in accordance with the Boyle-van't Hoff law, i.e., the volume increases linearly with 1/osmolality. This is the case in the osmolality range 110 to 1800 mosM. The deviation from the straight line at 77 mosM is due to osmotic lysis of vesicles since they were made at 100 mosM. It is interesting to see that in this system we observe no dead volume (osmotically inactive water). This is in contrast to the results of Bangham et al. [10], but the discrepancy can probably be explained either by differences in multilamellar and reverse phase evaporation vesicles or by the possibility that the present volume measurement method does not detect an osmotically inactive space.

One of the most common ways to determine vesicle volumes is through entrapment of a radioactive tracer. To compare the results obtained by the present method with radioactive techniques, reverse phase evaporation vesicles were made in the presence of [^{14}C]glucose. A typical result was as follows: (1) Trapped volume ([^{14}C]glucose) 12.7% or 20.4 μl/mg lipid. (2) Trapped volume (TEMPONE/chromium oxalate) 14.3% or 22.9 μl/ml lipid.

The agreement is reasonably good. However, the difference of 11% is larger than the uncertainty in the two actual measurements (1–2%). Additional experiments were carried out which indicated that the result based on the radioactive technique is too low because of leakage of trapped marker during the washing procedure. This explanation is also in accordance with results given by Roseman et al. [11]. They found about 10% leakage of [^{14}C]glucose from small unilamellar vesicles made from phosphatidylcholine during their washing procedure (4 h) while our results indicate 11% leakage from reverse evaporation vesicles during 22 h dialysis (and even higher leakage if cholesterol is excluded). Other explanations for the difference in measured volumes by the two techniques can be put forward, e.g. differences in self-broadening (spin exchange broadening) for spin labels within small compartments (trapped labels) compared with labels in free aqueous space at the same concentration (the reference sample). However, linewidth measurements did not support such an explanation in this particular experiment.

In conclusion, the ESR method has important advantages over the method based on isotope entrapment during vesicle formation. First, no separation of the vesicles from the medium is required with the former. Moreover, since the TEMPONE label is freely permeable to lipid bilayers, the method can be used to detect volume changes taking place subsequent to vesicle formation. Finally, so long as the vesicles are impermeable to the broadening agent at the time it is added, no errors are introduced by leakage of solute after the vesicle are formed.

These features of the ESR method make it highly suitable for studies of vesicle-vesicle fusion. As known from work by Papahadjopoulos and coworkers [12], small unilamellar vesicles made from phosphatidylserine are fused readily by Ca treatment. When Ca is added, 'chochleated cylinders' are made, and if these are EDTA treated, large unilamellar vesicles are formed [9]. Sun et al. [13,14] have studied the final size of these vesicles as a function of the incubation temperature. They found a maximum in size close to what they believe is the transition temperature for phosphatidylserine partially saturated with Ca. These properties can be studied by using trapped volume as the significant parameter.

Phosphatidylserine vesicles were made and treated as described above, and a typical result is given in Fig. 5. It appears to be extremely important to shake the 'chochleated cylinders' in the presence of EDTA in order to get the large unilamellar vesicles and a large trapped volume. Vortexing for 5 min at room temperature seemed to be optimal in our experiments with regard to obtaining high trapping and useful reproducibility. The resulting trapped volume was highly dependent on the Ca-incubation temperature. It appears that the final volumes are smaller by a factor 2–3 for incubation temperatures below approx. 9°C compared to the volumes above this temperature. For comparison the volume of unfused vesicles is given in Fig. 5. Very little variation is observed within each of the two domains below and above 9°C and a dramatic peak similar to that found by Sun et al. [14] was not observed. The procedure used in the present experiments is slightly different from that used by Sun et al. (We used higher lipid concentration and vortexing.) When ESR volume determinations were carried out on vesicles fused by the same procedure as that of Sun et al. (except for the difference in buffer), reproducibility of results was poor. No increase in trapped volume above 9°C was observed. Only about a 3-fold increase in total trapped volume compared to unfused vesicles was found in the temperature range 4–28°C.

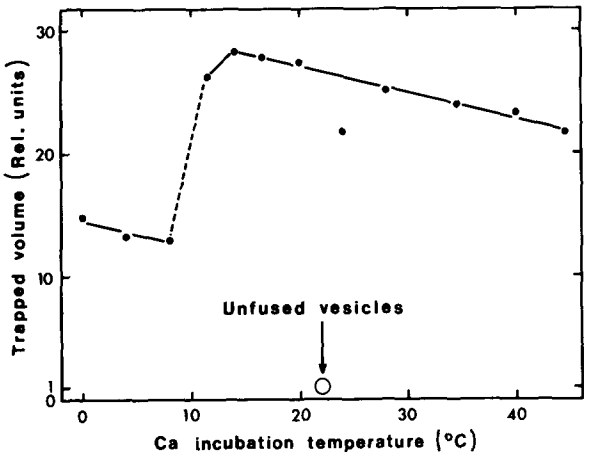

Fig. 5. Trapped volume of fused vesicles relative to unfused ones as a function of the Ca incubation temperature. Procedure: 70 μl SUV (4 mg phosphatidylserine/ml) was added to 70 μl 10 mM $CaCl_2$ in buffer, and both solutions equilibrated to a given temperature. After 1 h incubation at the same temperature, 7.9 μl 133 mM EDTA (pH adjusted to give pH 6.8 after Ca chelation) was added and the vial vortexed at room temperature for 5 min. 100 μl 150 mM chromium oxalate including 2.5 mM TEMPONE was added, and the ESR spectrum was taken within 10 min. Most of the points in the figure are the mean values of five samples from two separate experiments. The standard deviation in the points for the fused vesicles are roughly 4 in the given scale.

Conclusions

With regard to the fusion results presented here, the present volume measurements are qualitatively in agreement with those of Papahadjopoulos et al. [9,12]. Based on electron microscopy they found an enhanced Ca-induced fusion of small unilamellar vesicles made from phosphatidylserine at 24 and 37°C compared to the fusion at 0°C. Based on data on the endothermic transition of the phosphatidylserine vesicles and Na^+ efflux data they proposed that the change in fusion capabilities should take place somewhere in the range 5 to 12°C in agreement with our finding. However, it should be noted that even if fusion below about 9°C is less than above this temperature, it is, according to our data, not insignificant.

From the hypothesis that Ca-induced phase changes are necessary for fusion [12] it follows also that the degree of fusion should decrease at very high temperature. It might be that this is the reason for the observed decrease in trapped volume at increasing temperature above 10°C. However, instabilities and 'budding off' phenomena might equally well account for it.

The reason for the apparent differences between our results and those from Sun et al. [13,14] is not clear. The possibilities should be considered that some of the 'cochleated cylinders' in their experiments were not broken completely to large unilamellar vesicles. However, differences in lipid or buffer systems might play a significant role.

Other spin label methods can be used to measure trapped volumes under some circumstances. For example, an impermeable (or slowly permeable) label can be entrapped and, just prior to ESR measurements, ascorbic acid added to reduce any external label [15]. Like the method presented here, this procedure does not require the separation of vesicles from the medium, however we would like to point out that experiments like the one given in Fig. 4 or ones in which the volume changes rapidly cannot be carried out conveniently by use of any nonpermeable marker.

Permeable labels other than TEMPONE can also be employed in combination with broadening agents. However, it should be kept in mind that charged labels may be sensitive to potential and $[H^+]$ gradients across the membranes. An important advantage of TEMPONE is its narrow linewidth, which provides additional discrimination of spectral components. Thus, with the lipid and chromium oxalate concentration employed in Fig. 3, we were able to quantitate the volume of small unilamellar vesicles of phosphatidylserine with TEMPONE but not with TEMPAMINE, TEMPOL or TEMPO (unpublished observations).

More traditional methods for measuring trapped volume also present problems. Those associated with the trapped isotope method were discussed above. Measurements based on treatment with permeable and impermeable labels (e.g. ^3H_2O and [^{14}C]inulin) still require separation of the vesicles and precise determination of trapped external space. This can be time consuming and technically difficult in the case of small vesicles. These realities and the fact that the method based on TEMPONE is convenient, rapid, free from ambiguity, fairly accurate, and apparently applicable over a wide range of conditions makes it attractive for numerous future applications.

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