THE PREPARATION AND PROPERTIES OF LARGE LECITHIN BILAYER VESICLES

Thesis by

John N. Hurley

In Partial Fulfillment of the Requirements

For the Degree of

Master of Science in Chemistry

California Institute of Technology

Pasadena, California

September 1973

TABLE OF CONTENTS

ABSTRACT

Sonicated lecithin vesicles of an "average" diameter of 1200 Å were prepared. PMR studies show a higher phase transition temperature than that found for small (300 - 500 Å) vesicles. Osmotic pressure collapses the vesicle but does not change the PMR linewidth. This differs from the effect found in small vesicles. Upon addition of a cyclic polyether (cyclohexyl-15-crown-5) to a solution of large vesicles, the water diffusion rate is found to increase by a factor of 5 to 10. The internal water volume also increases significantly, suggesting that the crown compound is incorporated into the bilayer.

ACKNOWLEDGEMENTS

I would like to thank Professor Sunney I. Chan for his direction of this work. The members of the Chan group were very kind in teaching me enough about the theory and practice of NMR to be able to carry out this work. Special thanks must go to Mr. David Live who suggested the crown compound experiment and who supplied the sample of crown compound, and to Mr. Arthur Lau for performing the electron microscopy necessary. I would also like to acknowledge several helpful discussions with Dr. Michael Sheetz.

1. INTRODUCTION

This work is an extension of the work reported by Sheetz [1] and Sheetz and Chan [2]. The techniques used here are those used in their work.

Sheetz described two major-size classses of sonicated lecithin vesicles containing .1 M NaCl after elution from Sepharose columns, and he classified them as small (200 - 600 Å in diameter) and large vesicles (600 - 1200 Å and larger). Most of his work concerned characterization of small vesicles which seem to be the predominant size in most freshly sonicated lecithin solutions.

The preparation of large vesicles described here relies on one fact reported by Sheetz and Chan [2] that small vesicles prepared in low salt concentration will precipitate on, or otherwise not pass through, a Sepharose 4B column which has a running buffer of high salt concentration.

Sheetz [1] also showed that, in sonicated samples, more of the lecithin protons showed up in the NMR spectra of vesicles as one passed through the crystalline to liquid crystalline phase transition (Chapman transition [4]) of the lipid bilayer. It was also observed that there was a considerable difference in this property between small and large vesicles [2]. The large vesicles prepared by Sheetz had a

weighted average size of 900 - 1000 Å.

By preparing small vesicles in low salt, Sheetz [1] was able to show that it was possible to collapse the vesicles by addition of salt to the outside water. He determined the amount of H_2O inside by adding Mn^{++} and Ni^{++} to the outside water and thereby broadening the outside resonance. Sheetz also found that collapsing the vesicle had a great effect on the spectra of the lecithin protons. The most striking change was a broadening of the methylene and terminal methyl signals above the Chapman transition. Sheetz [1] was able to calculate that, for small vesicles, packing considerations of the vesicle structure might explain this behavior caused by osmotic pressure. His calculations also suggested that these packing considerations would not be so important in larger vesicles.

Sonicated phospholipid vesicles are being studied as model systems for membrane structure. The interaction of various membrane proteins with the lipid bilayer in membranes is a field that a considerable amount of work is being done in. The mechanism of active transport is still a major problem to be dealt with.

2. EXPERIMENTAL

2. 1. Procedure for the Preparation of Large Sonicated Lecithin Vesicles (1200Å).

Into each of two small test tubes were placed 2 ml of 2 mM NaCl in 99.5% D_2O (or H_2O for water diffusion studies) and .10 g l-∝-lecithin (dipalmitoyl, synthetic),(obtained from General Biochemicals and used without further purification). Each solution was then sonicated in an ice bath for 7 to 10 minutes at power level 4 on a Branson Sonifier with 1/8" microtip. (N.B. It seems to be very important that the sonication be of the gentle variety and not churning or turbulent.) These solutions were then centrifuged in a Sorvall RC-2 centrifuge for 10 minutes at 20,000 x g to remove suspended metal particles from the sonicator microtip and also any multilayer present. Some lipid will pellet to the bottom and some will rise to the top. The slightly cloudy solution between the pellet and the material on the top was carefully drawn off and made 10% in sucrose to aid layering on the column. This solution is carefully layered onto a Sepharose 4B column (95 cm x 2.5 cm). Before pouring this column, the gel had been equilibrated in the running buffer used. This running buffer contained:

.1 M NaCl

.02% NaN_3 (as a bacteriostat)

.01 M Tris base (Tris (hydroxymethyl) Amino Methane)

6 M HCl was added to bring the pH to 7.8.

Immediately upon layering the lecithin solution on the column, a precipitate of lecithin was formed just above the top of the column. As the column ran, this precipitate formed a white band at the top of the column. The column was run for about 12 hours to allow the large vesicles to move sufficiently down the column (about 60 ml eluted in this time). At this point the top 2 to 3 cm of Sepharose 4B gel was removed to get rid of this lecithin precipitate and to prevent further compression of the column packing (some compression of the gel cannot be avoided). On runs were this precipitate layer was small, the yield of large vesicles was good, whereas when a large precipitate layer was formed (one which compressed to gel significantly and plugged up the column) the yield of large vesicles was poor or nonexistent. The column was restarted and allowed to run until about 45 to 60 6 ml (200 drop) fractions had been collected. An elution curve for the lecithin vesicles is determined by measuring the light scattered at 300 nm on a Beckman DU Spectrophotometer.

As soon as possible the fractions containing the lecithin vesicles are concentrated in a 10 ml Amicon pressure dialysis apparatus with a UM-10 membrane. The fractions are combined and concentrated to a total volume of about 2 ml. This concentrated solution is then dialysed against 2 volumes of 2 mM NaCl in D_2O (or H_2O) at $4^{\circ}C$. This solution is removed

from the dialysis tubing and centrifuged for 10 minutes at 20,000 x g at 4°C to remove any bilayer that has formed during the preparation. This solution is stored at 4°C until needed.

2. 2. Electron Microscopy of Vesicles by Negative Staining.

Parlodion coated grids (200 mesh) freshly coated with carbon are used. A drop of the vesicle sample is placed on the grid for 30 seconds, blotted, and washed with 2 or 3 drops of 2% phosphotungstic acid (pH 7.2). A small drop of phosphotungstic acid is then left on the grid for 30 seconds, the grid is then blotted with filter paper and allowed to dry. Size standards are made in an identical manner with a 1000 Å polystyrene bead solution. Electron micrographs were then taken of these grids.

2. 3. NMR Temperature Studies.

Spectra of solutions of large vesicles prepared in D_2O with 2 mM NaCl both inside and outside were taken in the Fourier Transform mode on the Varian HR-220 NMR spectrophotometer. Spectra were run at temperatures between ambient (19°C) and 61°C. As a proton area standard, a capillary (external) of chloroform (diluted with deuterochloroform to about 1:5) which was saturated with the free radical 2,2-diphenyl-1-picrylhydrazyl was used.

2. 4. Osmotic Pressure.

To a solution of large vesicles prepared in 2mM NaCl in H_2O was added additional NaCl so that the outside NaCl concentration was varied between 0.1 M NaCl and 0.5 M NaCl. Sufficient Mn SO_4 was added to make the external concentration .03 M in Mn^{++}. Spectra were taken of the H_2O resonances of these solutions on the HR-220 in the CW mode at ambient probe temperature. To a solution of large vesicles prepared in D_2O containing 2mM NaCl additional NaCl was added so that the outside NaCl concentration was varied between 0.1 M NaCl and 0.5 M. Spectra were taken of the lecithin resonances of these solutions on the HR-220 in the Fourier Transform mode at 47° to $49^{\circ}C$.

2. 5. Addition of Cyclohexyl-15-crown-5 to Large Vesicles.

To a sample of large vesicles prepared in H_2O containing 2mM NaCl was added .015 ml of cyclohexyl-15-crown-5.[5] A 220 MHz NMR spectrum of this sample was taken immediately in the CW mode at $19^{\circ}C$. A chloroform standard was used as above. The spectrum was repeated on the same sample after 2 hours. The sample was then stored at $4^{\circ}C$. The spectrum was again repeated 48 hours after addition of the crown compound. (N.B. The sample in this experiment was .35 ml in volume. Therefore the sample was about .1 M in crown compound.)

3. RESULTS

3. 1. Large Vesicles Prepared.

The elution curve from the Sepharose 4B column is shown in figure 1. It consists of a single peak which was concentrated down and used for these experiments.

3. 2. Size Distribution.

For spectroscopic purposes the "average" size that is relevant is the size of the vesicle that the "average" lecithin molecule is in. That is, since large vesicles contain much more lecithin than smaller ones, the size distribution must be weighted according to the surface area. The results of such a size distribution which was determined from the electron micrographs is found in Table 1. The distribution is rather broad, but one should note the absence of significant numbers of small vesicles. The weighted average size is about 1200 Å.

3. 3. Phase Transition Study of Large Vesicles.

Figure 2 shows the relative amounts of choline methyl and combined methylene and terminal methyl protons observed as

FIGURE 1

Optical density at 300 nm of 6 ml eluant fractions from Sepharose 4B column (2.5 x 95 cm) in running buffer for vesicles containing 2mM NaCl.

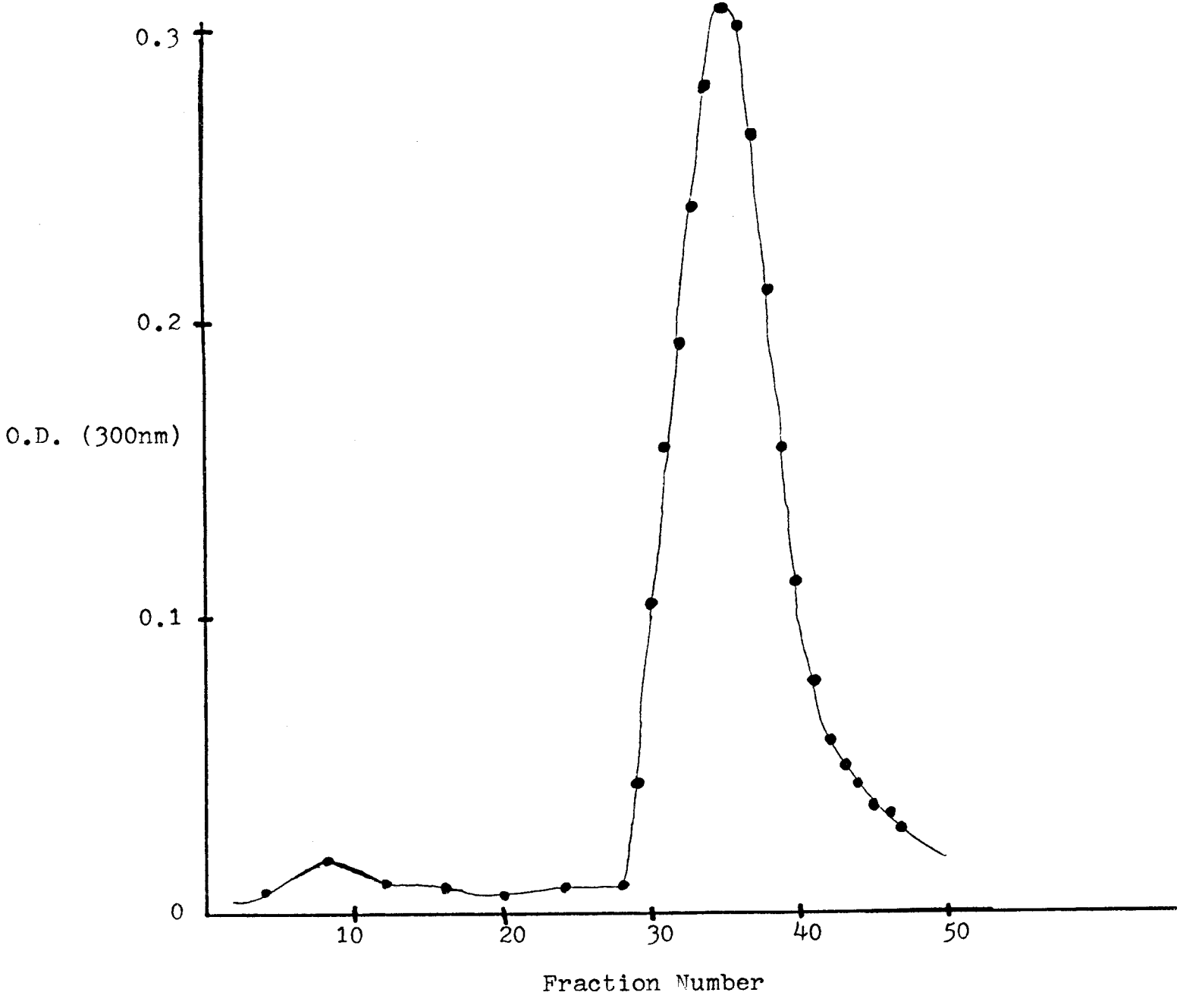

0.3
0.2
0.1
0
O.D. (300nm)
10
20
30
40
50
Fraction Number

TABLE 1. Percent by Weight of Lecithin in Different Size Vesicles in the Large (1200 Å) Vesicle Preparation

Diameter of Vesicle (Å)	Percent Lecithin by Weight
<500	1
500 - 700	15
700 - 900	21
900 - 1100	29
1100 - 1300	18
1300 - 1500	4
>1500	11

Figure 2

Relative amounts of choline methyl protons observed (●) and the relative amounts of combined methylene and terminal methyl protons observed (x) as a function of temperature.

Methylene and Terminal Methyl (x)
64
60
56
52
48
44
40
36
Temperature (°C)
Choline Methyl (●) (scale arbitrary)

a function of increasing temperature in large vesicles prepared in 2 mM NaCl in D_2O. The phase transition determined by NMR is very broad and is higher than that reported for small vesicles by Sheetz and Chan [2]. The phase transition does not appear to be complete below about 48°C. Thermal measurements of the phase transition (DTA) were not done due to the very dilute concentration of lecithin in these large vesicle preparations (0.5% or less).

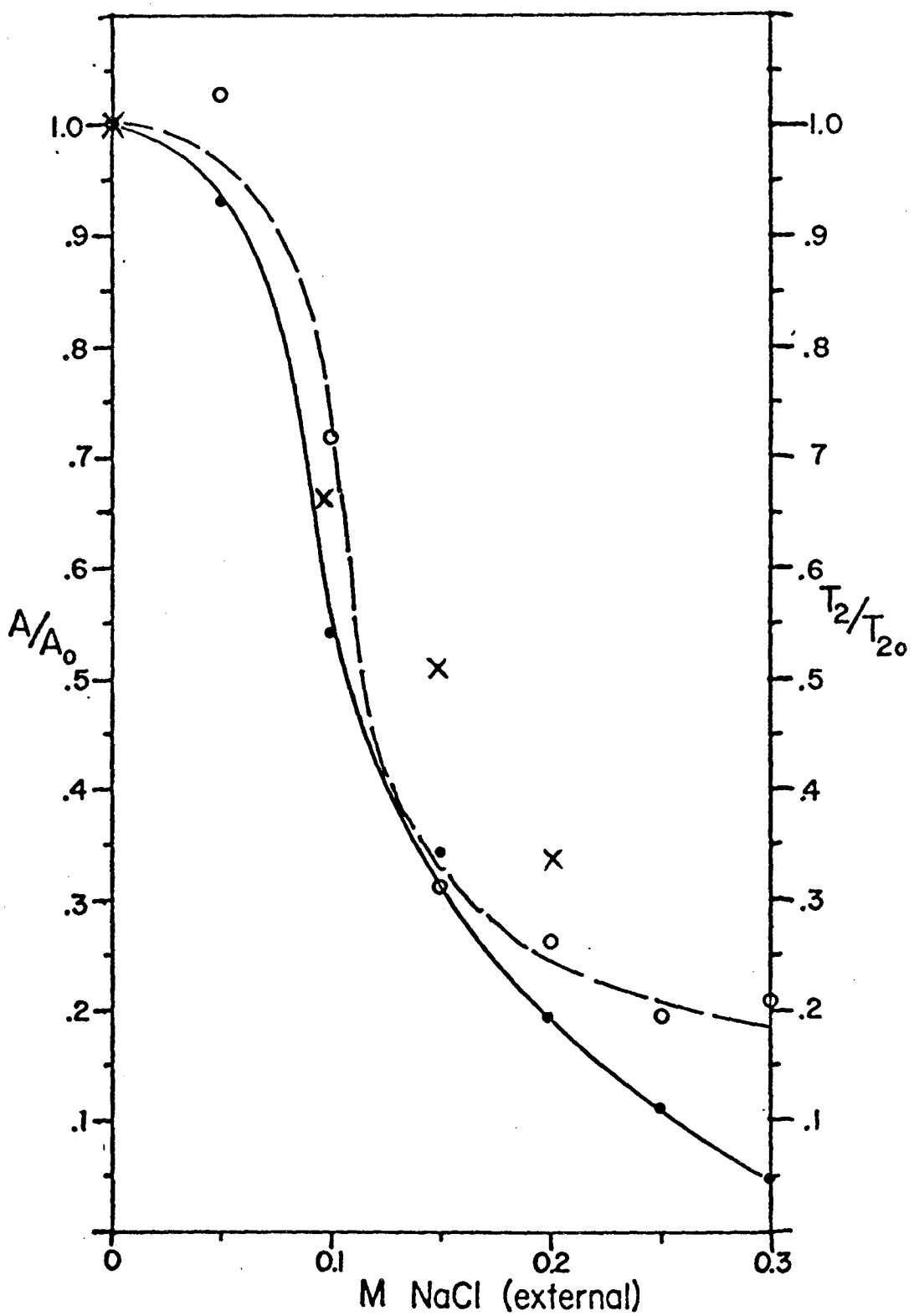
3. 4. Effect of Osmotic Pressure on NMR Spectra of Large Vesicles.

When the external salt concentrations were raised in samples of large vesicles prepared in H_2O, the water resonance of the internal water was seen to decrease in a way similar to that found by Sheetz [1] for small vesicles. The results of the experiments on large vesicles are plotted along with Sheetz' data in Figure 3. This shows that the vesicles are able to collapse due to an osmotic pressure gradient across the vesicle.

When vesicles prepared in 2 mM NaCl in D_2O were used in this osmotic pressure experiment, there was no change in the linewidth of either the choline methyl or the methylene resonances as additional outside salt was added. This is considerably different from the result for small vesicles found by Sheetz [1]

FIGURE 3

Sheetz (1) has plotted the ratio of the area (●) or the T_2 (o) of the internal water resonance from small (300 - 500 Å) vesicles in the presence of added external NaCl to the area or the T_2 respectively of the internal water resonance from small vesicles in an isotonic solution versus the external NaCl concentration. To this plot is added the data from the current experiment for comparison. The ratio of the area (x) of the internal water resonance from large (1200 Å) vesicles in the presence of added external NaCl to the area of large vesicles in 2mM NaCl with .03 M $MnSO_4$ added is plotted against the added external NaCl concentration.


1.0
.9
.8
.7
.6
.5
.4
.3
.2
.1
A/A₀
T₂/T₂₀
0
0.1
0.2
0.3
M NaCl (external)

where increased osmotic pressure caused a considerable broadening of the lecithin resonances.

An adequate study of the effect of osmotic pressure on the fusion of vesicles was not done. However, higher concentrations of NaCl did tend to precipitate the lecithin faster than lower concentrations. The fusion process does not seem to effect the spectra of those vesicles remaining in solution.

3. 5. Effect of Cyclohexyl-15-crown-5 on Water Diffusion and Internal Volume of Large Vesicles.

Immediately after adding the crown compound, the inside water resonance increased in linewidth from 8 Hz to 40 Hz. Since the crown compound binds Mn^{++} in solution, and since the crown compound is present in considerable excess, the apparent five-fold increase in the rate of water diffusion across the vesicle (see Sheetz [1]) is probably slightly greater due to reduced effect of Mn^{++} on linebroadening.

By integrating the internal water resonances before and after addition of the crown compound, the internal water resonance is seen to increase by a factor of about 2.3 upon adding the crown compound. If one assumes that this increase is due to incorporation of the crown compound into the bilayer

and not to any fusion processes, this corresponds in an increase in the average diameter by a factor of 1.3. The spectra of this sample remains the same over a period of 2 days which argues against any sort of vesicle fusion hypothesis to explain the increased internal water volume. Also, this argues against Mn^{++} transport across the bilayer as an explanation of the linewidth broadening mentioned above since this also remains unchanged with time.

4. DISCUSSION

The present experiments lend support to Sheetz' calculations [1] which predict that collapse of small vesicles, which are highly strained due to packing considerations, causes great changes in the motion of the lipids in the vesicle thereby causing changes in the NMR spectral linewidth. These calculations also predict that large vesicles, which are thought not to be very highly strained, might not be greatly disturbed by collapse by osmotic pressure but retain their basic structure and spectral parameters.

The Chapman transition for the large vesicles is much broader and occurs at a much higher temperature than either bilayer sheets or small vesicles. The structural implications of this fact are not obvious but its importance in planning experiments with large vesicles is clear.

The effect of the crown compound on water diffusion in large vesicles is quite interesting. The experiment was originally done to see if the partially lipid soluble crown compound might have Mn^{++} transport properties. That possibility has been fairly well ruled out. Much more work on the characterization of this system by concentration, temperature, and osmotic pressure studies is necessary.

5. REFERENCES

1. M. P. Sheetz, Ph.D. Thesis, California Institute of Technology (1972).

2. M. P. Sheetz and S. I. Chan, Biochemistry 11, 4573 (1972).

3. C. H. A. Seiter and S. I. Chan, in press.

4. D. Chapman, R. M. Williams, and B. D. Ladbrooke, Chem. Phys. Lipids 1, 445 (1967).

5. C. J. Pedersen, J. Amer. Chem. Soc. 89, 7017 (1967).