MODIFICATION OF MEMBRANE SURFACES WITH CARBOHYDRATES: AN APPROACH FOR STABILIZATION DURING FREEZING AND DRYING

Thesis By
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I would like to dedicate this work to my parents whose love and support has made all things possible throughout my life. I would also like to acknowledge the friendship and support of Spiros Courellis. I have never known a finer person or friend.

Finally, I would like to acknowledge the love and support of Boon Hui Tay and thank her for the encouragement that she has provided during the writing of this work. Without that support, this task would have been much less joyful than it has been.

ACKNOWLEDGEMENTS

As I review my time spent at Caltech, the most prominent feature is the quality of people I have had the opportunity to interact with and develop scientific and personal relationships with. Foremost among these are the people I have worked with in the Baldeschwieler group. I also would like to acknowledge the help and support provided by several visitors to the group over the past several years. These include Dr. Richard Colton from the Naval Research Labs who provided the equipment and directions for use of a film balance at Caltech and provided a valuable contact for further monolayer work conducted in collaboration with the Naval Research Labs. I also would like to acknowledge Dr. Mary Roberts, Taka Osonai, Ian McKay, Dr. Dick Hoekstra, and Dr. Wilton Vannier for help and support at various times during my stay at Caltech.

I have been fortunate also in having had the opportunity to interact with several investigators in related fields throughout the past. These include Dr. Richard Kondrat at the University of California, Riverside who performed the Mass Spectroscopy on all of the compounds utilized in studies reported here. I must also thank Dr. Bill Barger at the NRL for providing useful information from monolayer studies using several of the derivatives. Foremost, I wish to thank John and Lois Crowe for their support and interest in this project throughout the years. In particular I

wish to thank John Crowe for his openness and friendliness in discussing much of this work.

In terms of financial support, I am grateful to the National Institute of Health training grant program, which has provided me with support throughout my stay at Caltech and allowed me to travel to various meetings throughout the United States for the purpose of presenting my own work as well as keeping up on the advances made in other laboratories. The experience has been invaluable throughout my graduate career.

Finally, I must acknowledge the support and direction provided by John Baldeschwieler. This project is a direct consequence of his ability to recognize exciting and innovative research topics and extract from them the important features necessary to build an interesting research project. It was in following his advice to enter this area that I was propelled into what has turned out to be the most challenging, exciting, and creative period of my life. I have yet to see where that path may lead me. Each day I spend on research endeavors related to it are filled with intriguing and exciting observations and results. I will always be grateful for that advice and for the freedom to pursue new ideas and topics as they arose.

Abstract

A new class of molecules possessing amphipathic character was prepared. These compounds possessed a hydrophobic region capable of intercalation into a lipid bilayer, a hydrophilic linker group capable of extending beyond the surface of a membrane , and a carbohydrate attached at the end of this linker group. These features of this class of compounds permitted their direct incorporation into vesicle formulations and hence the direct examination of interactions occuring in the dry state between carbohydrates and lipid groups in such vesicle membrane systems.

Samples of treated vesicle preparations were subjected to freezing and thawing as well as to direct dehydration via lyophilization. Under these conditions, the stability and integrity of the membrane was examined via several spectroscopic techniques.

Through these studies of systems in which a carbohydrate is directly bound to a membrane surface, it was possible to determine a defined ratio, independent of solution and concentration effects, at which carbohydrates can afford protection to dehydrated membranes. In addition, the interactions responsible for conferring the protection were determined. It was found that direct intercalation of the carbohydrates into a membrane interface preserves the membrane

structure and organization that is normally observed in the presence of water. This behavior prevents the phase transitions, lipid phase separations, and fusion phenomena that normally compromise dehydrated membrane systems. This phenomena is directly related to the amount of carbohydrate that is present and the structure of the carbohydrate that is used. These results indicate that the partitioning behavior of the carbohydrates at the interface is of prime importance in determining the effectiveness in this regard.

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INTRODUCTION

When I began this project in January of 1986, there were many reports present in the literature, which suggested that "something" interesting was happening when membranes were placed in contact with carbohydrates in solution or in the dry state. What was not clear was what actually was occuring. Most of the information generated during the early part of the 80's came from the laboratory of John and Lois Crowe at the University of California at Davis. It was John Crowe who as much as 15 years earlier had opened the door to a new era in cryobiology through his studies of organisms that were capable of surviving conditions of low water activity. Crowe went on to investigate the nature of this phenomena to discover that carbohydrates were intimately involved in the protection of the organism's membranes against the dehydration induced damage. This relationship was dramatically demonstrated through pioneering work using liposome formulations as model membrane systems. Many of the techniques pioneered by Crowe in his laboratory have subsequently formed the basis for work carried out in other laboratories involved in cryobiology, including much of the work reported here.

The major thesis of this dissertation arose from a consideration of the facts available in January of 1986. It was known that carbohydrates were involved in the mechanism of cryoprotection. It was also known that the protection was

afforded via some form of interaction at the vesicle surface, potentially involving hydrogen bonding and water replacement. Our goal was to further examine this phenomena by attaching carbohydrates directly to the surface of a vesicle and examining whether such a system exhibited similar behavior to that reported for free carbohydrates. In this modification it would thus be possible to remove from consideration the carbohydrate that was not immediately present at the interfacial region and therefore was not interacting with the membrane directly. From an argument based on availability, the placement of the carbohydrate at the membrane surface would potentially enhance the likelihood of interaction because the local concentration could be increased and complex formation enhanced. This argument, as we were soon to find out, would depend on several conditions:

(1) The bulk carbohydrate and bulk phase properties were not critical for determining the extent of cryoprotection (i.e., only the carbohydrate at the interface produced cryoprotection).

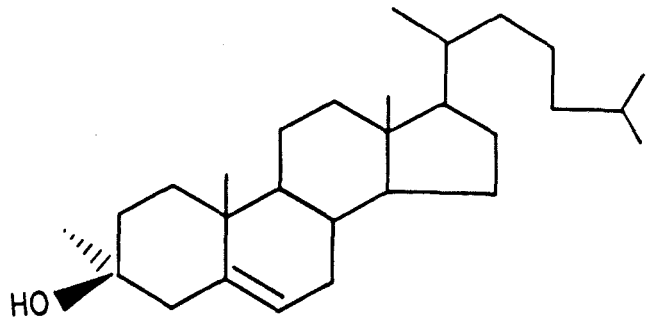
(2) The attached carbohydrate demonstrated the same properties as untethered carbohydrates with respect to the ability to afford cryoprotection.

(3) The use of compounds interacting directly with the bilayer did not disrupt the bilayer integrity or alter bilayer structure adversely due to detergent-like properties.

The compounds that were prepared to meet these criteria are depicted in Fig. 1 and will be referred to throughout the remainder of the text as the derivatives.

Fig. 1. Structure of the derivatives used in studies discussed throughout the remainder of the text. (A) Cholesterol (B) Triethoxycholesterol (TEC) (C) Triethoxycholesterol-Galactose (TEC-Gal) (D) Triethoxycholesterol-Maltose (TEC-Mal).

A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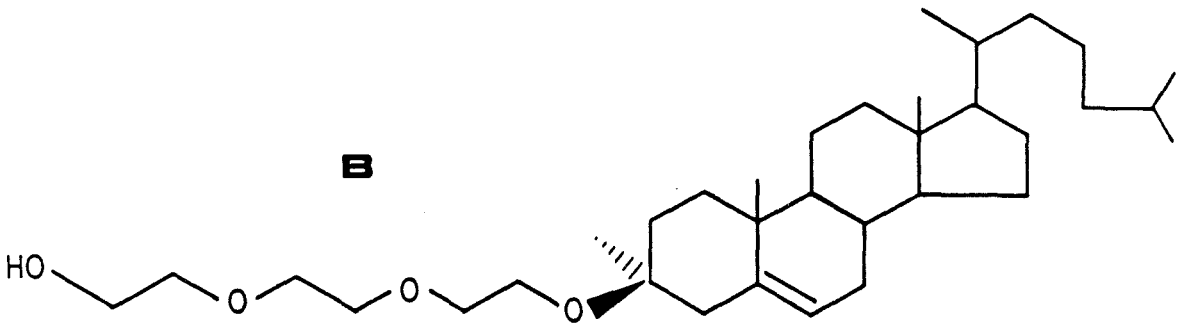
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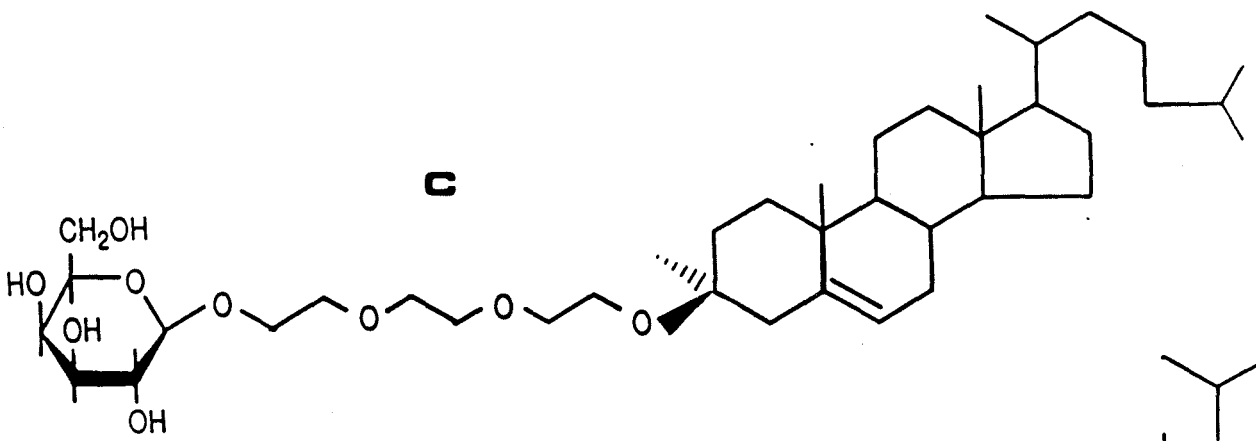
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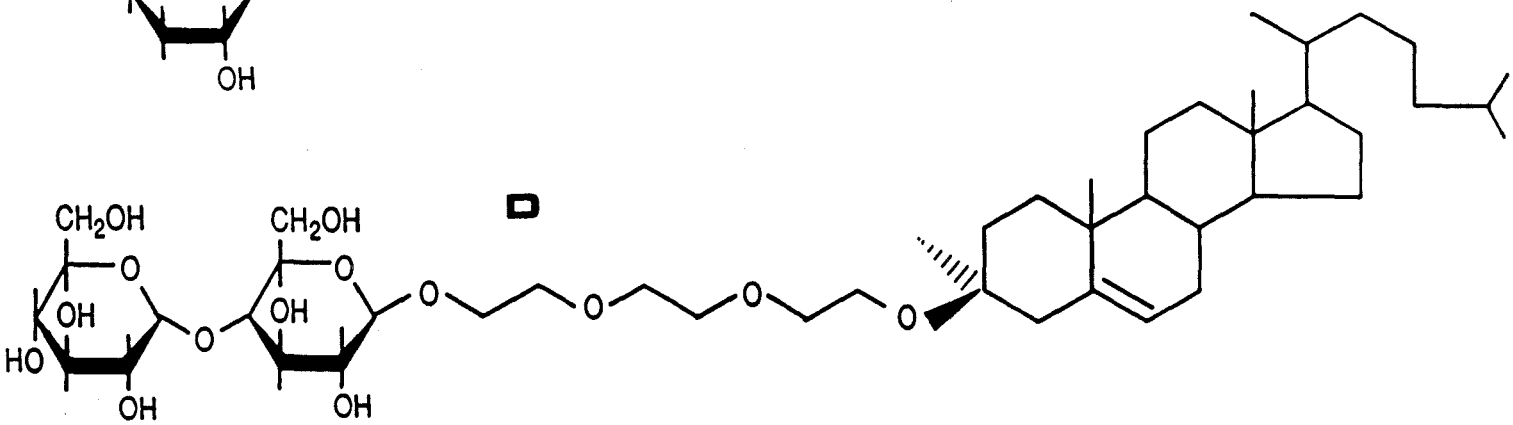
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Chapter One represents an article published in *Biochimica et Biophysica Acta* in 1988. It details the solution properties exhibited when the carbohydrate derivatives utilized in this study were combined with lipid dispersions of DOPE, DOPC, and DPPC. This article indicated that strong interactions between the derivatives and lipid vesicles were occurring in aqueous solutions that are normally associated with alterations produced by cryoprotectants in the aqueous phase. In Chapter Two the cryoprotective capacity of the derivatives and the nature of the interaction in the aqueous phase was examined further. This was subsequently followed with research on the capacity of the derivatives to afford protection to membranes in which water had been directly removed from the system (Chapter Three).

In Chapter Four, the nature of the pure derivatives in aqueous solution (ie, without additional lipids) was examined. The bilayer forming capacity of these compounds demonstrated here, explained in part some of the alterations presented in the preceding chapters, and established the capacity to incorporate large portions of the derivatives into a bilayer structure without inducing destabilization of the bilayer. In Chapter Five, the protective action and requirements for protection of vesicles dried in the presence of the carbohydrate derivatives was examined. Chapter Five represents the culmination of the work related in the previous chapters in which the actual mechanism for what is occurring at the vesicle interface in the presence of carbohydrates is presented. This work was carried out in partial

collaboration with John and Lois Crowe at the University of California at Davis.

All of the information and data discussed in Chaps. 1-5 has been presented during Biophysical Society meetings in Phoenix, Arizona, New Orleans, Lousiana, and Cincinnati, Ohio from 1987-1989. This material has also been published in abstract form in each of the Proceedings from these meetings.

I have included the synthesis and characterization section for the derivatives in the Appendix. While the compounds produced are novel and unique in character, the methodology utilized in producing them is well known and documented in the literature. For this reason, publication as a separate article was not deemed appropriate. The work is presented here in full to provide characterization of the compounds utilized in the studies reported in the preceding chapters.

CHAPTER 1

Modification of Lipid Phase Behavior with Membrane-Bound Cryoprotectants

Abstract

Several derivatives of cholesterol containing oxyethylene headgroups with and without a terminal galactose have been synthesized in order to examine the effects of immobilizing a cryoprotectant at a membrane surface. In this work, we have studied the ability of the triethoxycholesterol (TEC) and triethoxycholesterol galactose (TEC-Gal) derivatives to modulate the phase behavior of phosphatidylcholine and phosphatidylethanolamine membranes. Methods of fluorescence polarization, ^{31}P-NMR, and freeze-fracture electron microscopy were employed to monitor these changes in lipid phase behavior. Fluorescence polarization data demonstrated the ability of the derivatives to fluidize gel state and rigidify liquid-crystalline state phosphatidylcholines in a manner similar to that observed for cholesterol. Unlike cholesterol, however, the T_m of dipalmitoylphosphatidylcholine (DPPC) was reduced in a concentration-dependent manner with each of the derivatives. Freeze-fracture electron microscopy and ^{31}P-NMR of DOPE dispersions indicate an increase in the lamellar to hexagonal phase-transition temperature on the order of 10-20 C^o above room temperature for mixtures with 20 mol% of the derivatives. These results are discussed in terms of the properties exhibited by compounds such as carbohydrates, which are known to serve as cryoprotectants for synthetic and biological membranes.

Introduction:

The role of carbohydrate structure and function with respect to membrane stability has been and continues to be an area of intense investigation. In recent years, much discussion has been devoted to the question of interactions of carbohydrate with membrane surfaces. In particular, interactions of carbohydrate with phospholipids have been examined.[1,2,3] The effects of such interactions include modification of phospholipid phase behavior and stabilization of phospholipid vesicle structure under conditions of freeze-drying and freeze-thawing.[4,5,6,7,8]

Cryoprotectants such as dimethyl sulphoxide, glycerol, ethylene glycol, and urea have been known and utilized for many years for the cryopreservation of biological materials.[9] Interest in carbohydrates for the same purposes was spurred by the discovery over 10 years ago that certain organisms capable of surviving in a dehydrated state for many years produce large amounts of trehalose, a non-reducing disaccharide of glucose.[10,11] Strauss and co-workers and Crowe et al. have since demonstrated that the addition of trehalose or sucrose to liposomes of synthetic or biological origin enables these structures to retain their morphological and functional characteristics upon freezing and thawing, or dehydration.[5,6,7] It was found for example, that at concentrations of 0.3 g/g membrane, trehalose preserved the integrity of Ca^{2+} transporting microsomes that had been dehydrated (these structures do not normally survive dehydration).[10] In terms of effectiveness,

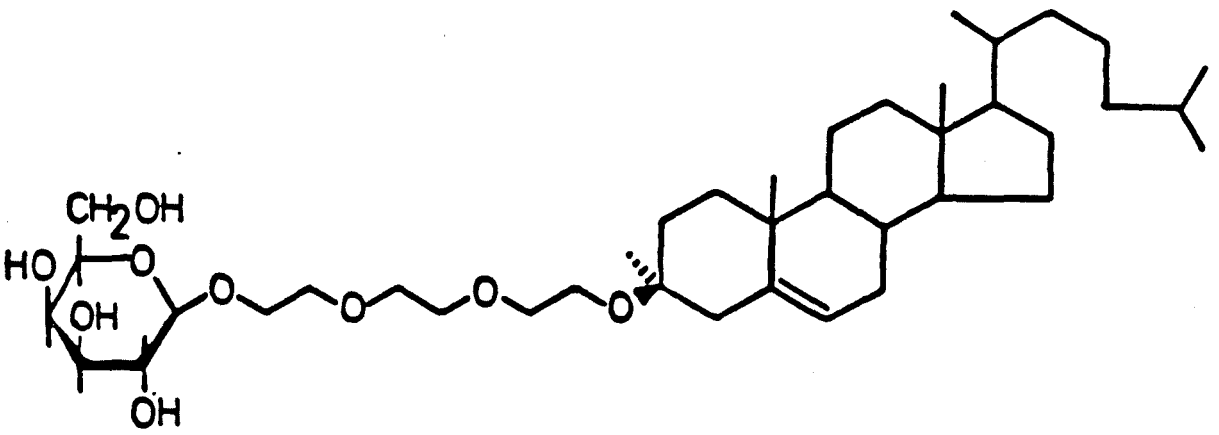
trehalose has been shown to be three times more effective than sucrose and several times more effective than other cryoprotectants for these particular membranes.[10] Several postulates have been proposed to explain the mechanism of carbohydrate action. These models are based primarily on evidence demonstrating the ability of carbohydrates to modulate phase behavior.[1,2,3,4,5,6,7,8] The data include observations of the ability of certain carbohydrates to (a) reduce the phase-transition temperature in SUV[4] and (b) to stabilize hexagonal-phase-forming lipids in the lamellar phase.[11]

An intriguing question arising from this work is whether carbohydrates directly attached to the membrane surface elicit even more pronounced effects (i.e., modification of phase behavior and stabilization) than carbohydrates free in solution. In such a system, the carbohydrate moiety would be localized at the membrane surface, and so potentially enhance the interactions required for membrane stabilization. Selective modification of a membrane surface could be accomplished with relatively small amounts of the cryoprotectant compared to the concentrations of free carbohydrate required to preserve membrane integrity in dehydrated or frozen systems.

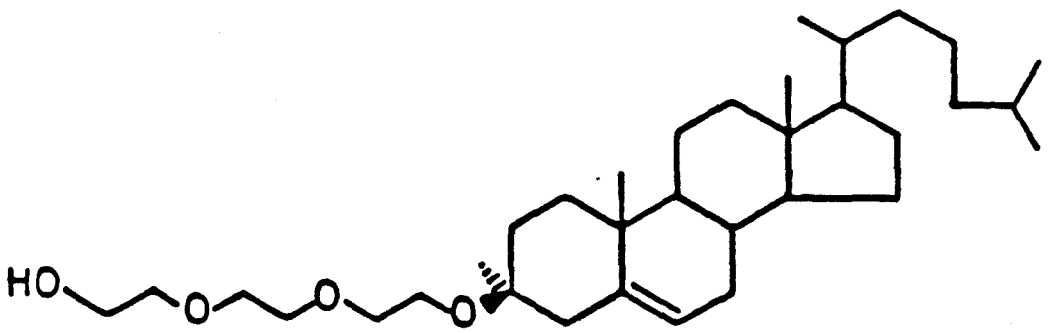
We have prepared the cholesterol derivatives (Fig.1) for the purpose of examining and optimizing carbohydrate-lipid interactions. The compounds consist of a carbohydrate (galactose) placed at a variable distance from the hydrophobic portion of the molecule via a hydrophilic oxyethylene unit of variable length. These features permit (a) incorporation of the molecule into a

bilayer via a steroid anchor, (b) variation in position and mobility of the carbohydrate moiety for maximization of the potential interactions and (c) variation in the structure of the carbohydrate. The derivative without the sugar (B) allows direct examination of the requirements for a carbohydrate structure.

Fig. 1. Structure of cholesterol derivatives.
(A) 3,6,9-trioxaoctan-1-β-D-galactosylcholesteryl-3β-ol (TEC-Gal)
(B) 3,6,9-trioxaoctan-l-ol-cholesteryl-3β-ol (TEC)



(A) TEC-GAL



(B) TEC

For initial studies, we have examined derivatives where $n=3$. These compounds are designated as TEC-Gal (Fig. 1, A) and TEC (Fig.1, B). The synthesis is described in the Materials and Methods. The incorporation of these derivatives into phospholipid membranes composed of dipalmitoylphosphatidylcholine (DPPC), dioleoylphosphatidylcholine (DOPC), or dioleoylphosphatidylethanolamine (DOPE) are shown to modify the lipid phase behavior. These effects have been demonstrated to be directly related to the cryoprotective action exhibited by a compound.[12] Several methods have been employed to monitor these alterations.

Fluorescence anisotropy of DPH was utilized to monitor alterations in the membrane ordering and gel to liquid-crystalline phase-transition temperature of DOPC and DPPC respectively. ^{31}P-NMR and freeze-fracture electron microscopy were used to assess changes in the lamellar to hexagonal phase-transition temperature of DOPE. The results are compared to the influence of cholesterol on membrane structure and fluidity. A comparison of the effects of trehalose and other cryoprotectants on these properties is also made.

Materials and Methods

Materials

DOPC, DOPE, and DPPC were purchased from Avanti Polar Lipids, (Birmingham, AL). Cholesterol was purchased from Sigma. l,3,5-Diphenylhexatriene was purchased from Aldrich.

Methods

Preparation of poly(oxyethylene) derivatives of cholesterol. Samples of the mono-, di-, and triethoxy derivatives of cholesterol were prepared by refluxing the appropriate poly(ethylene glycol) with cholesteryl-ρ-toluenesulfonate in dioxane for 2-3 hrs.[13,14] The dioxane was removed by rotary evaporation and the resulting material was taken up in water. The milky aqueous layer was extracted with diethyl ether. The organic layer was subsequently washed with a 10% aqueous sodium carbonate solution, dried over anhydrous sodium carbonate, and removed by rotary evaporation. The monoethoxy and diethoxy derivatives yielded solids, whereas the triethoxy derivative formed a liquid-crystal at room temperature. All materials were purified by column chromatography on silica gel, and by recrystallization from methanol for the solids. Samples were characterized by TLC, NMR, mass spectra, and melting point determinations.

Preparation of carbohydrate derivatives. The appropriate cholesterol derivative was dissolved in benzene and placed in a round-bottom flask fitted with an addition funnel. To

this was added silver oxide, iodine, and powdered molecular sieves (4 Å, dried at 100° C). Acetobromo-α-D-galactose, which had been dissolved in benzene, was added to the stirred mixture via the addition funnel.[15] The sugar was added dropwise over a period of 1 hr. The molar ratio of siver oxide/iodine/sterol/sugar was 2:1:1:2. The mixture was stirred at room temperature in the dark for 3-7 days. The progress was followed by TLC on silica gel. The mixtures were filtered, and the organic solvent was removed by rotary evaporation. The acetate protected glycolipids were purified by column chromatography on silica gel. Samples were characterized by TLC, NMR, mass spectra, and melting points.

The acetate groups were removed by dissolving the compounds in methanol, to which was added a small quantity of sodium methoxide (0.05 M). The reaction progress was followed by TLC and litmus. The mixture was neutralized at the end of the reaction by treatment with an Amberlite exchange resin. Samples were characterized by TLC, NMR, mass spectra, ultraviolet spectroscopy, and melting points. Specific ($FeCl_3{}^{o}6H_20$,Naphthol) as well as nonspecific (H_2S0_4) stain reagents were used to develop TLC plates and confirm the presence of sterol and carbohydrate moieties in the glycolipids.

^{31}P-NMR. Samples for ^{31}P-NMR were prepared by drying lipids in 10mm (o.d.) NMR tubes under nitrogen followed by thorough drying under vacuum for 7-12 hrs. to remove trace solvent. Samples were hydrated with NTE buffer (100 mm NaCl/10 mm Tris-HCl/0.2 mm EDTA (pH 7.4)), and an aliquot of the $^{2}H_20$ analog of this buffer

used for signal lock. Lipid concentrations were kept constant at 56 mM. Samples were dispersed by vortex mixing for 10-30 min, or freeze/thawing with vortexing. The duration of vortexing varied according to the phase behavior of the mixture. Samples that formed the hexagonal phase were vortexed for longer periods to assure complete dispersion. In general, samples were prepared at least 1 hr. prior to use.

All samples were examined using a Bruker 500 MHz NMR with broad-band proton decoupling (for TEC-Gal) or inverse gated decoupling (for TEC). The two decoupling methods did not alter the observed spectra. Free induction decays were collected from 500-1000 transients by employing an 8.0 ms 90^{o} radiofrequency pulse with a 3 s interpulse delay and 50 kHz sweep width. For kinetic studies at various temperature intervals, a 15.0 ms 90^{o} radiofrequency pulse with a 0.04 s interpulse delay was utilized to permit rapid accumulation of spectra. An exponential multiplication corresponding to 100 Hz line-broadening was applied to the free induction decay prior to Fourier transformation.

Fluorescence spectroscopy. Lipid samples were dried down from solvent under a stream of nitrogen and subsequently under vacuum for 6-12 hrs. to remove traces of organic solvent. All samples were hydrated with NTE buffer (100 mM NaCl/10 mM Tris-HCl/0.2 mM EDTA (pH 7.4)) above the phase-transition temperature. The final lipid concentration of each sample was 1.0 mmol/ml. Samples were vortexed for 10 min above the phase-transition temperature. An aliquot of 2 ml of a 2 mM DPH solution (in

tetrahydrofuran) was added to the samples, which were subsequently equilibrated for 2 hrs. above the phase-transition temperature. Values for polarization were collected using an SLM-4800 spectrofluorometer (λ_{ex}= 357nm, λ_{em} = 430 nm). A T-format was utilized for placement of the photomultiplier tubes with a long pass filter in the emission channel that cut off light below 430 nm. The temperature in the sample chamber was controlled with a circulating waterbath and monitored using a thermocouple inserted into the cuvette. Samples were stirred continuously during measurement via a magnetic stirrer to prevent the settling of multilamellar vesicles. The effects of scattered light on the measured anisotropies was found to be negligible. Polarization and anisotropy were calculated using the following relationships:

$$P = \frac{I_{||} - I_{\perp}}{I_{||} + I_{\perp}}$$

$$r = \frac{2P}{3-P}$$

Electron microscopy: Samples from ^{31}P-NMR were examined using freeze-fracture electron microscopy. The initial

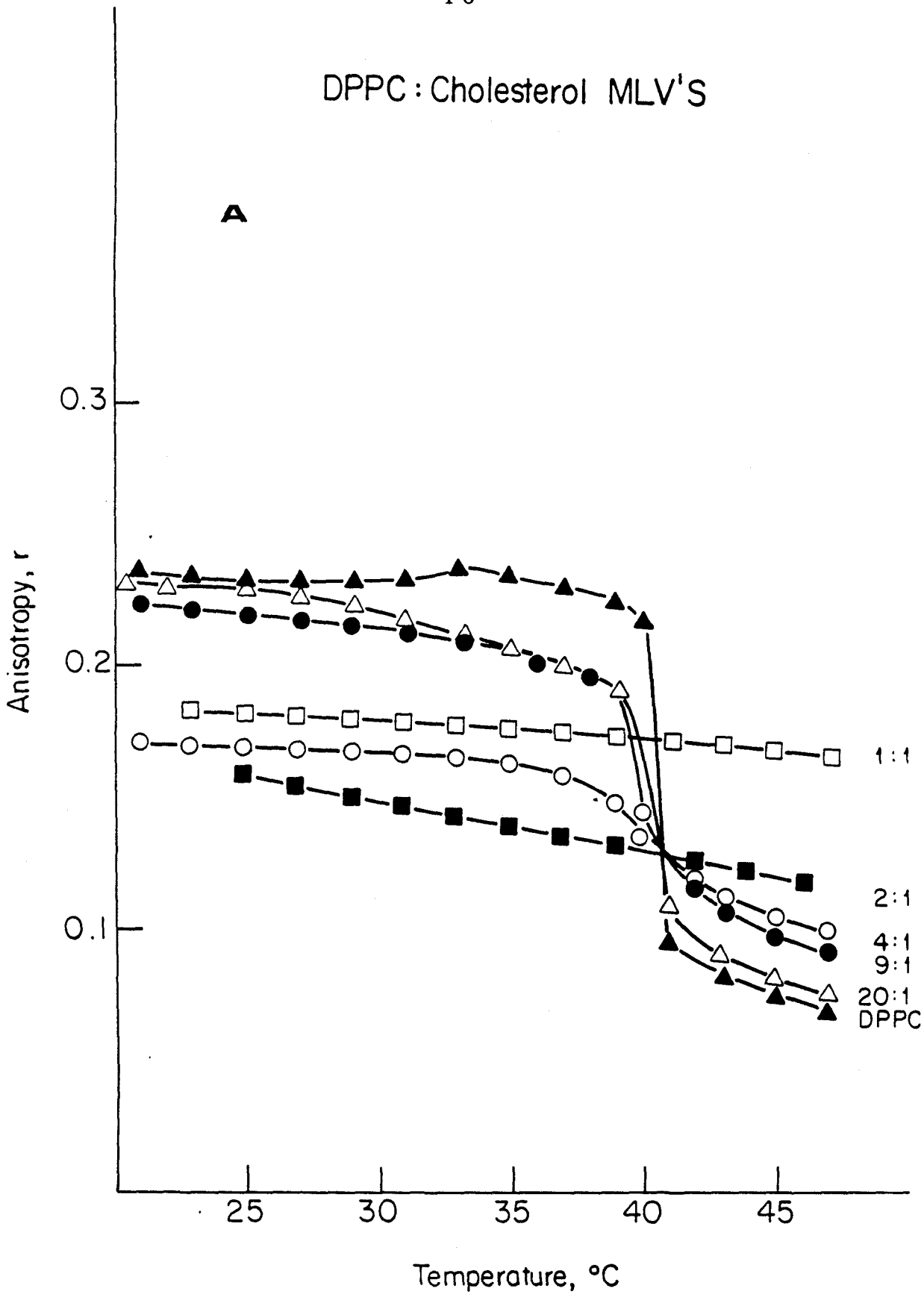
lipid concentration of each was 56.1 mM. Aliquots were spun down for 10-15 s to pellet the lipid. The pellet was then resuspended in a 30% glycerol solution of the buffer of equal volume. Aliquots of these samples were frozen in liquid freon from room temperature and subsequently fractured at -100^{o}C in a Balzer's apparatus, and etched for 1 min and shadowed with Pt/C. All samples were examined with a Philips EM 201 electron microscope operating at 80kV.

Results

Fluorescence spectroscopy

Fluorescence anisotropy of DPH is commonly utilized to monitor the gel-to-liquid crystalline phase transition as well as to estimate membrane fluidity.[16,17,18] Fig. 2A illustrates the temperature dependence of DPH anisotropy for DPPC MLV with increasing cholesterol content up to 50 mol%. Consistent with previous observation by workers using various techniques (NMR, ESR, fluorescence anisotropy), the addition of cholesterol results in a decrease in the measured anisotropy in the gel state and an increase in the measured anisotropy in the liquid-crystalline state.[19,20,21] These changes have been associated with a disordering effect of cholesterol on the gel state and an ordering effect on the liquid-crystalline state.[22,23,24,25] As the diagram also illustrates, the phase-transition temperature of the lipid in the presence of cholesterol remains relatively constant, although it is broadened to the point of being undetactable at a lipid-cholesterol ratio of 2:1.

Fig. 2. Anisotropy vs. temperature of (A) DPPC:Cholesterol (B) DPPC:TEC-Gal (C) DPPC:TEC MLV DPPC; 20:1, 9:1; 4:1, 2:1, 1:1.


DPPC : Cholesterol MLV'S
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Anisotropy, r
1:1
2:1
4:1
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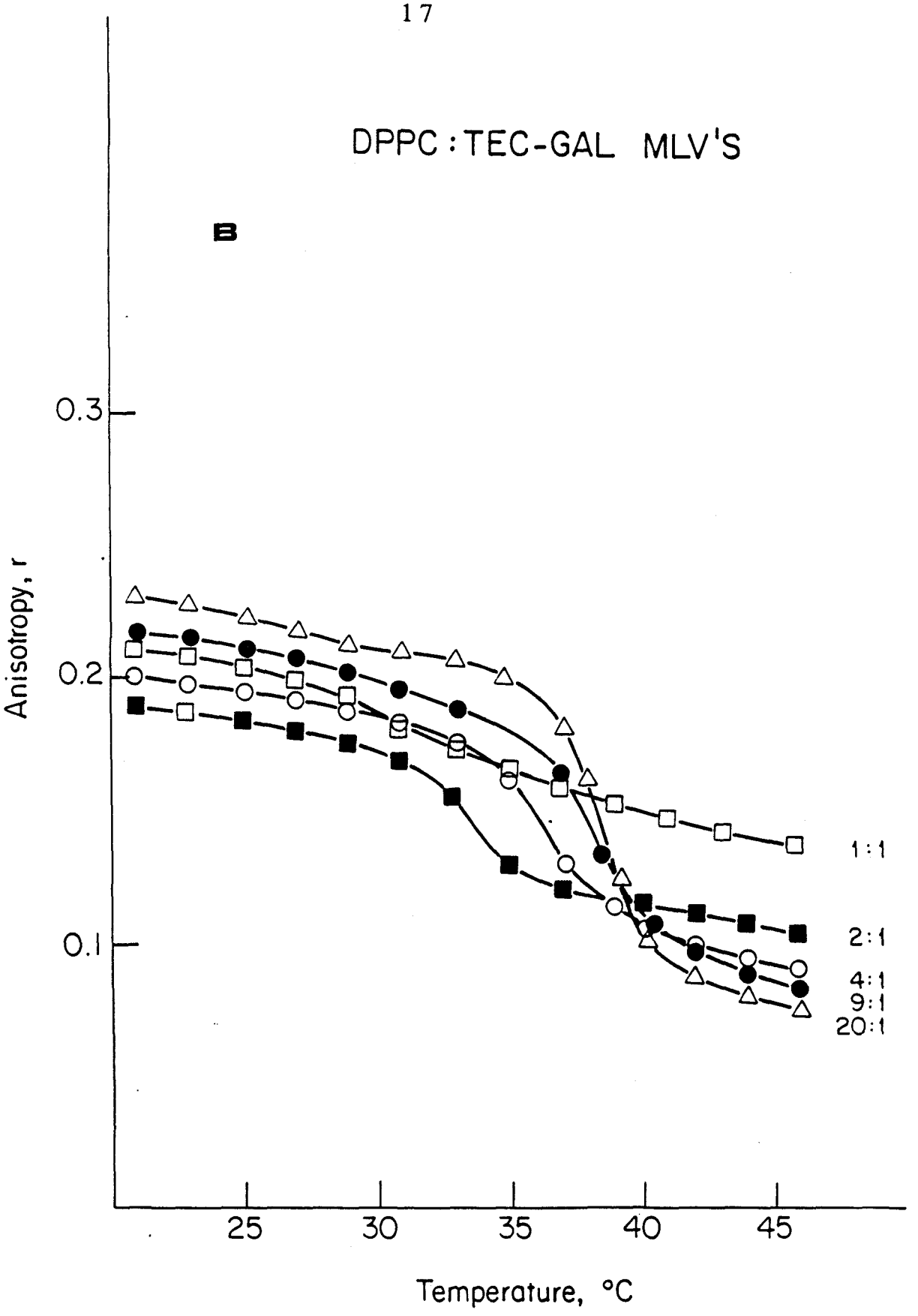
20:1
DPPC
25
30
35
40
45
Temperature, °C


DPPC : TEC-GAL MLV'S
B
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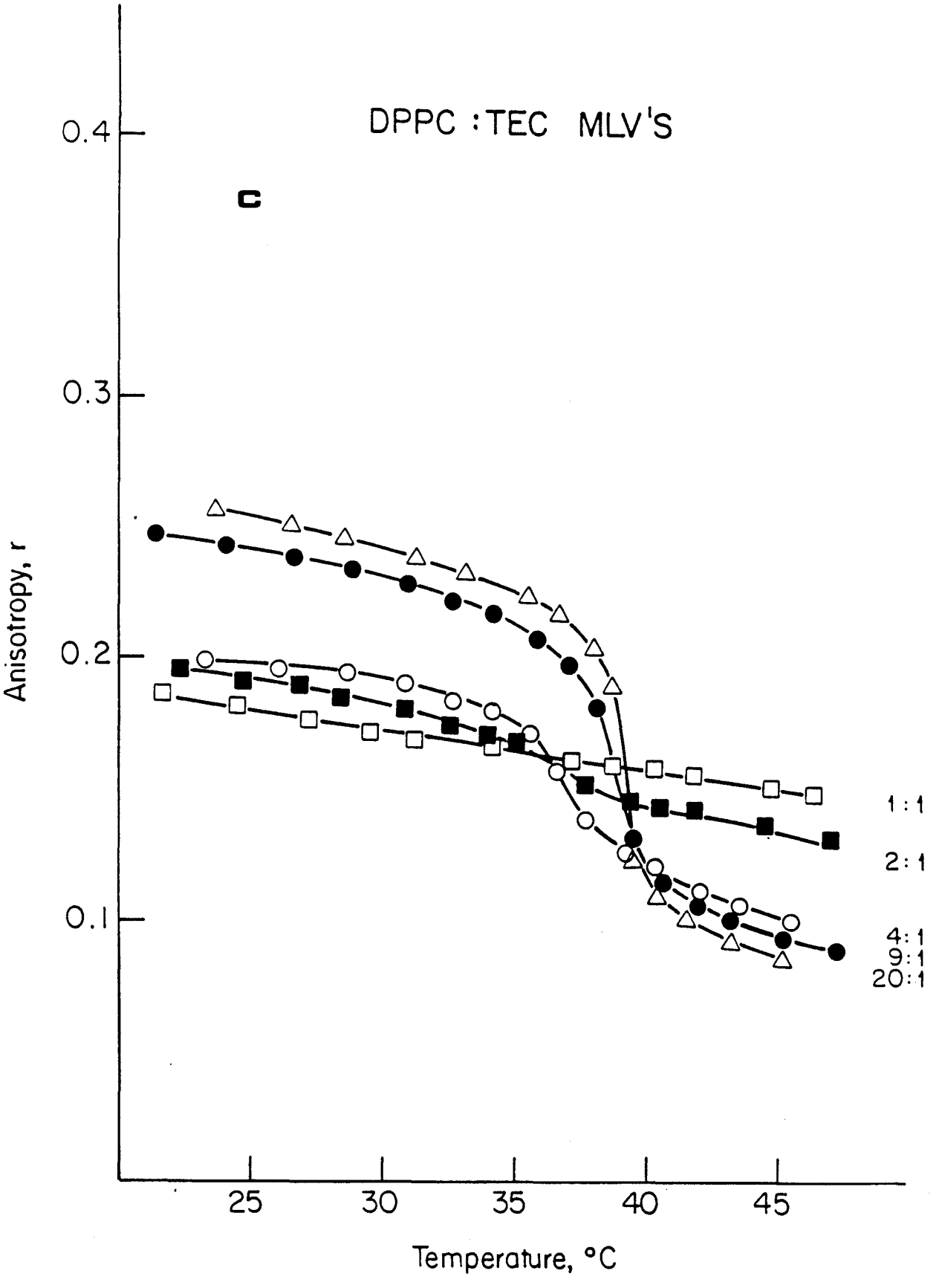
20:1


DPPC : TEC MLV'S
C
0.4
0.3
0.2
0.1
Anisotropy, r
25
30
35
40
45
Temperature, °C
1 : 1
2 : 1
4 : 1
9 : 1
20 : 1

The behavior of cholesterol may be compared with that obtained for the sterol derivatives (Figs. 2B, C). Addition of both TEC and TEC-Gal cause a reduction in the measured anisotropy of DPH in the gel state and an increase in the liquid-crystalline state. The effect is similar to that observed for cholesterol, but is reduced in magnitude. In contrast to cholesterol, both derivatives induce a progressive reduction in the phase-transition temperature with increasing concentration. In both cases, the reduction is linear with concentration and corresponds to a decrease of approx. 0.17-0.20^{o} C per mol% sterol. Furthermore, the transition is still detected at concentrations of 33 mol% for TEC and 50 mol% TEC-Gal, where no transition is observed for mixtures with cholesterol. Table I summarizes the effects on the phase-transition temperature observed in each case.

S.D. are in parentheses. Chol, cholesterol.

Mol% sterol	Phase-transition temperature (°C)		
	Chol	TEC	TEC-Gal
0	40.5	40.5	40.5 (0.7)
5	40.3	39.3	39.0 (0.7)
10	39.8	38.8	38.5 (0.7)
20	40.0	37.0	37.2 (0.6)
33	-	35.1	33.6 (0.6)
50	-	-	3l.9 (0.4)

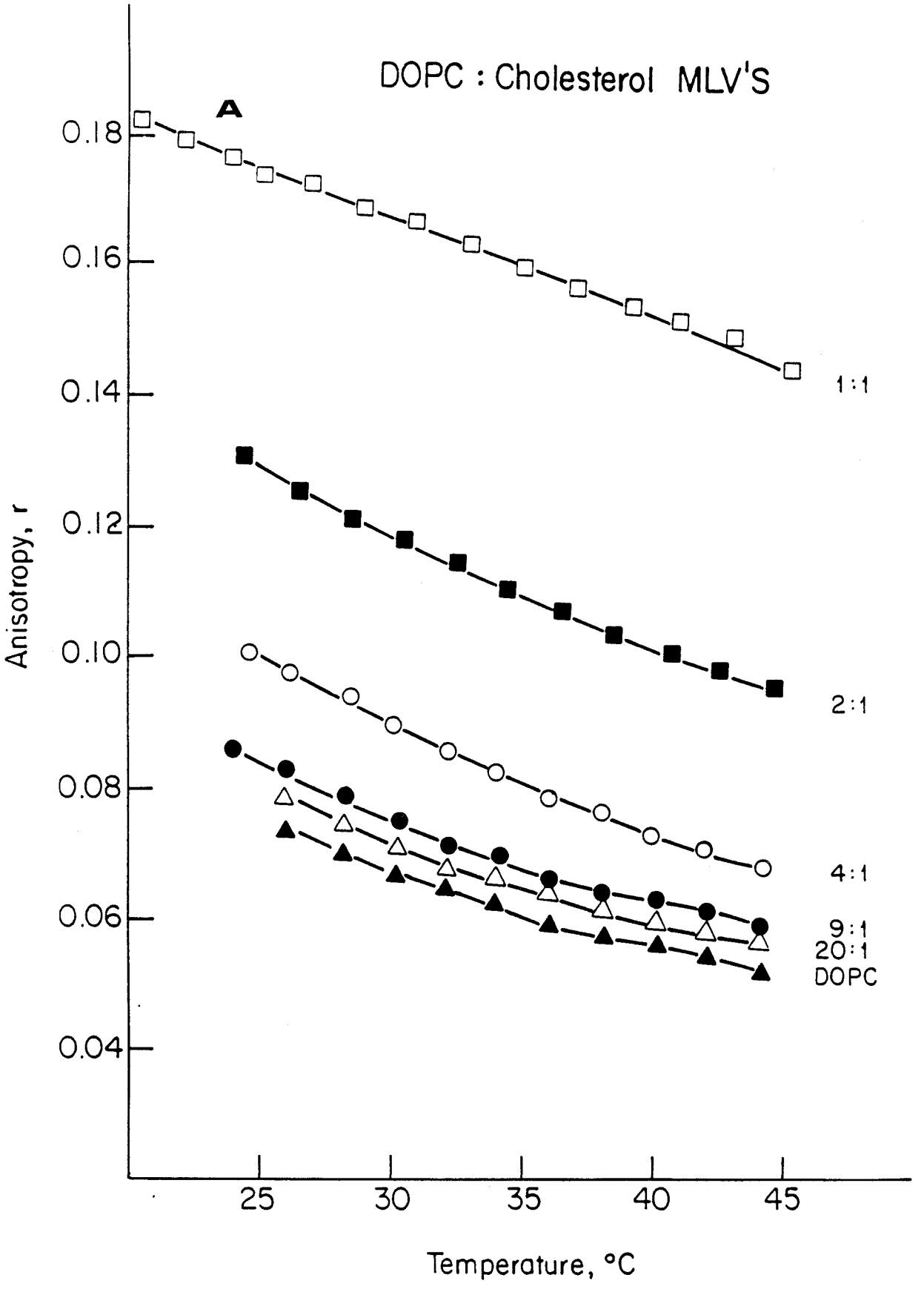
Table I: Phase-Transition Temperature as a function of sterol content for DPPC:Cholesterol, DPPC:TEC and DPPC:TEC-Gal.

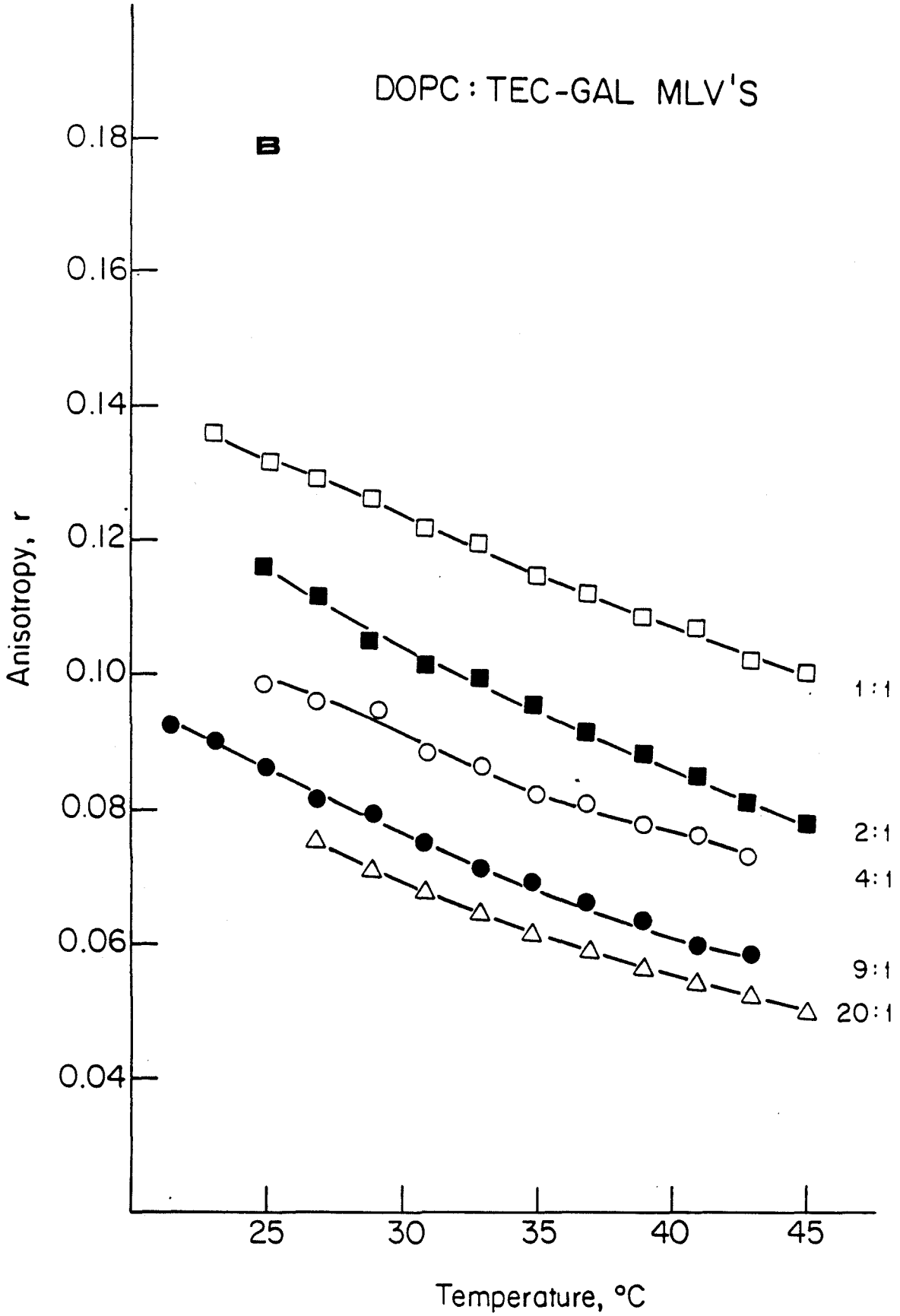
To examine further the effect of the derivatives on the ordering of the lipid in the liquid-crystalline state, anisotropy measurements were made on DOPC MLV containing varying proportions of the sterols. DOPC undergoes the gel-to-liquid-crystalline transition at -22°C. [26] At room temperature and above, it exists in a highly fluid state characterized by low anisotropy values for DPH. With increasing cholesterol, however, the anisotropy increases (Fig.3A). The magnitude of the increase offers an estimate of the ordering effect that cholesterol exerts on the bilayer.[27,28] For TEC and TEC-Gal, similar ordering was observed, although slightly reduced in magnitude (Figs. 3B and C; Table II). These results are consistent with those reported by Lyte and Shinitzky for egg PC:Cholesteryl phosphorylcholine mixtures and with Lee's results for DOPC:Cholesteryl hemisuccinate mixtures.[27,29]

Mol % sterol	Anisotropy		
	Chol	TEC	TEC-Gal
0	0.060	0.060	0.060
5	0.063	0.053	0.060
10	0.067	0.056	0.066
20	0.079	0.080	0.081
33	0.106	0.087	0.091
50	0.160	0.120	0.112

Table II: Anisotropy of DPH at 37°C as a function of sterol content for DOPC:Cholesterol, DOPC:TEC and DPPC:TEC-Gal.

Fig. 3. Anisotropy vs. temperature of (A) DOPC:cholesterol (B) DOPC:TEC-Gal (C) DOPC:TEC MLV. DOPC; 20:1; 9:1; 4:1; 2:1; 1:1.


DOPC : Cholesterol MLV'S
A
0.18
0.16
0.14
0.12
0.10
0.08
0.06
0.04
Anisotropy, r
1:1
2:1
4:1
9:1
20:1
DOPC
25
30
35
40
45
Temperature, °C


DOPC : TEC-GAL MLV'S
B
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0.12
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Anisotropy, r
1 : 1
2 : 1
4 : 1
9 : 1
20 : 1
25
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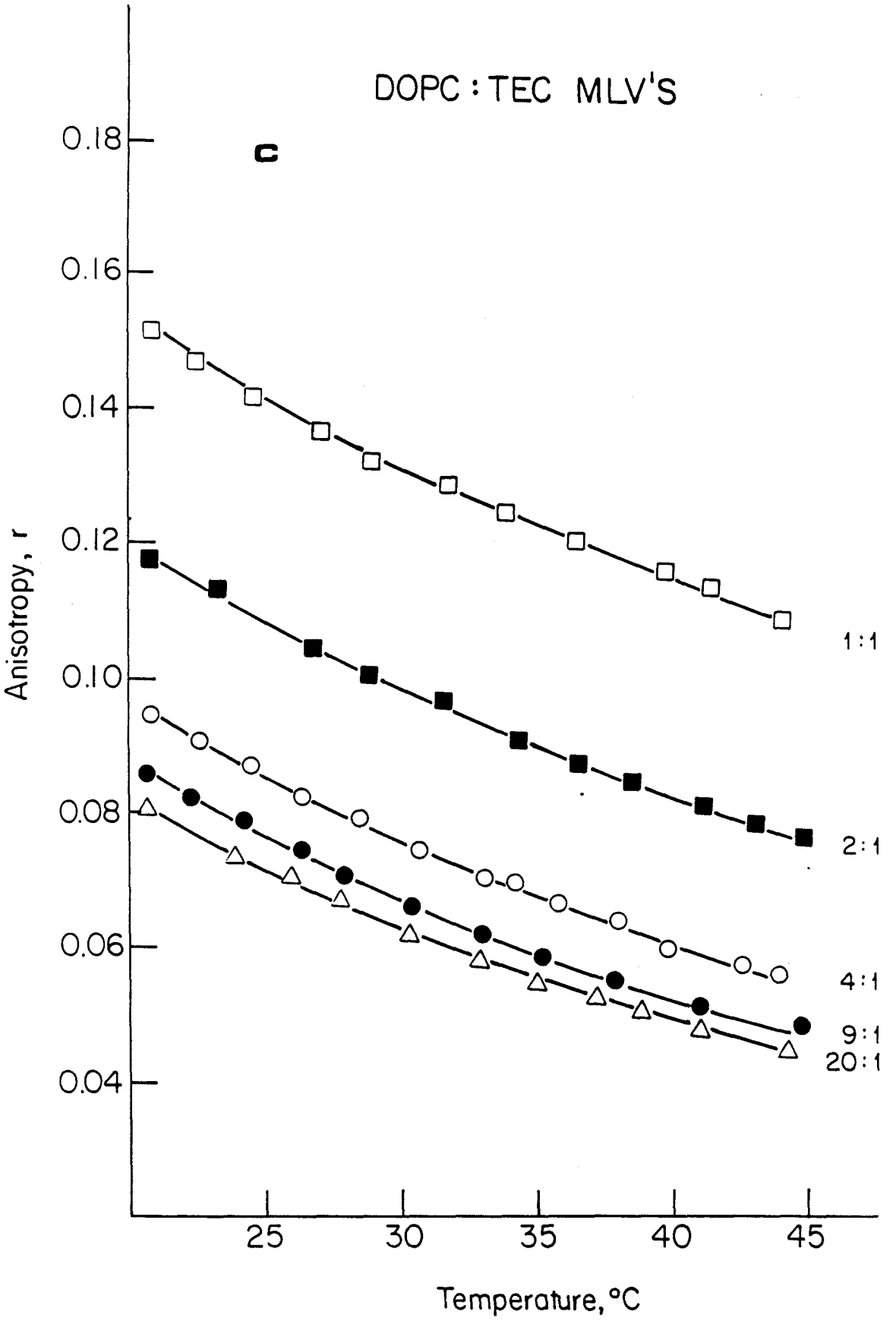
35
40
45
Temperature, °C


DOPC : TEC MLV'S
c
0.18
0.16
0.14
0.12
0.10
0.08
0.06
0.04
Anisotropy, r
1:1
2:1
4:1
9:1
20:1
25
30
35
40
45
Temperature, °C

^{31}P-NMR spectroscopy

^{31}P-NMR is frequently employed to study the phase behavior of lipids in natural and artificial systems.[30,31] In particular, ^{31}P-powder patterns offer a convenient way to monitor transitions between lamellar, hexagonal, cubic, or micellar phases. For lipids in a lamellar phase, restricted motion of the lipids results in only partial averaging of the large chemical shift anisotropy of the ^{31}P nucleus. The basic features of the spectrum generated under these conditions include a low field shoulder and high field peak separated by $\Delta\sigma$ =40ppm. Lipids in the hexagonal phase can undergo additional motional averaging due to lateral diffusion around the aqueous channels that the tubules enclose. The line-shape generated under such conditions has reversed symmetry in comparison to the lamellar spectra, and is narrowed by a factor of 2. Finally, lipids in vesicles, micelles, or phases, such as cubic or rhombic, allow isotropic (complete) averaging of the tensor. This occurs due to diffusional motions of the lipids over a wide variety of orientations on a time scale of at least 10^{-8} cm^2/s.[32,33] Under these conditions, a narrow symmetric ^{31}P-spectrum is generated.

Unsaturated phosphatidylethanolamines normally adopt a hexagonal phase (H_{II}) arrangement at a given temperature and pH.[33,34] The exact temperature at which this transition occurs for a particular type of PE is sensitive to hydration, acyl chain composition, pH and the presence of particular solutes.[35,36,37,38] The propensity of PE to adopt the hexagonal phase arrangement may

be explained in terms of the fact that PE is normally poorly hydrated, an effect that is enhanced by the potential for strong hydrogen bonding among the headgroups.[35,36,37,38]

DOPE undergoes the lamellar-to-hexagonal transition at approx. 10-18° C.[35] Incorporation of cholesterol in the membrane promotes the formation of the hexagonal phase. This effect has been demonstrated by ^{31}P-NMR and is attributed to the packing constraints imposed by the steroid due to its relatively small poorly hydrated head group and large hydrophobic body.[39]

Fig. 4 illustrates the effect of various mole ratios of TEC and TEC-Gal on the phase behavior of DOPE. In contrast to cholesterol, these derivatives increase the temperature at which the lamellar phase exists. For both TEC and TEC-Gal, the minimum derivative concentration required to stabilize DOPE fully into a lamellar phase at room temperature is 20 mol%. To assess the extent of the stabilization at this composition, the temperature dependence of the spectra was examined. For the DOPE/TEC mixture (4:1) (Fig. 5). DOPE begins to enter the hexagonal phase at approx. 30° C. The transition is complete by 35° C. DOPE/TEC-Gal mixtures remain in the lamellar phase at temperatures up to 45° C. There is little variation in the spectra at this temperature for at least 1 hr.

Heating above 45° C, however, induces the formation of hexagonal and isotropic components of the spectra (Fig. 6). Upon cooling the sample back to room temperature, spectra characteristic of the lamellar phase are regenerated. It thus appears that the lamellar phase is the equilibrium phase at room temperature for these compositions.

Fig. 4. ^{31}P-NMR powder patterns for DOPE:TEC dispersions at 24^{o} C. (A) 10 mol% (B) 20 mol% (C) 33 mol% TEC.

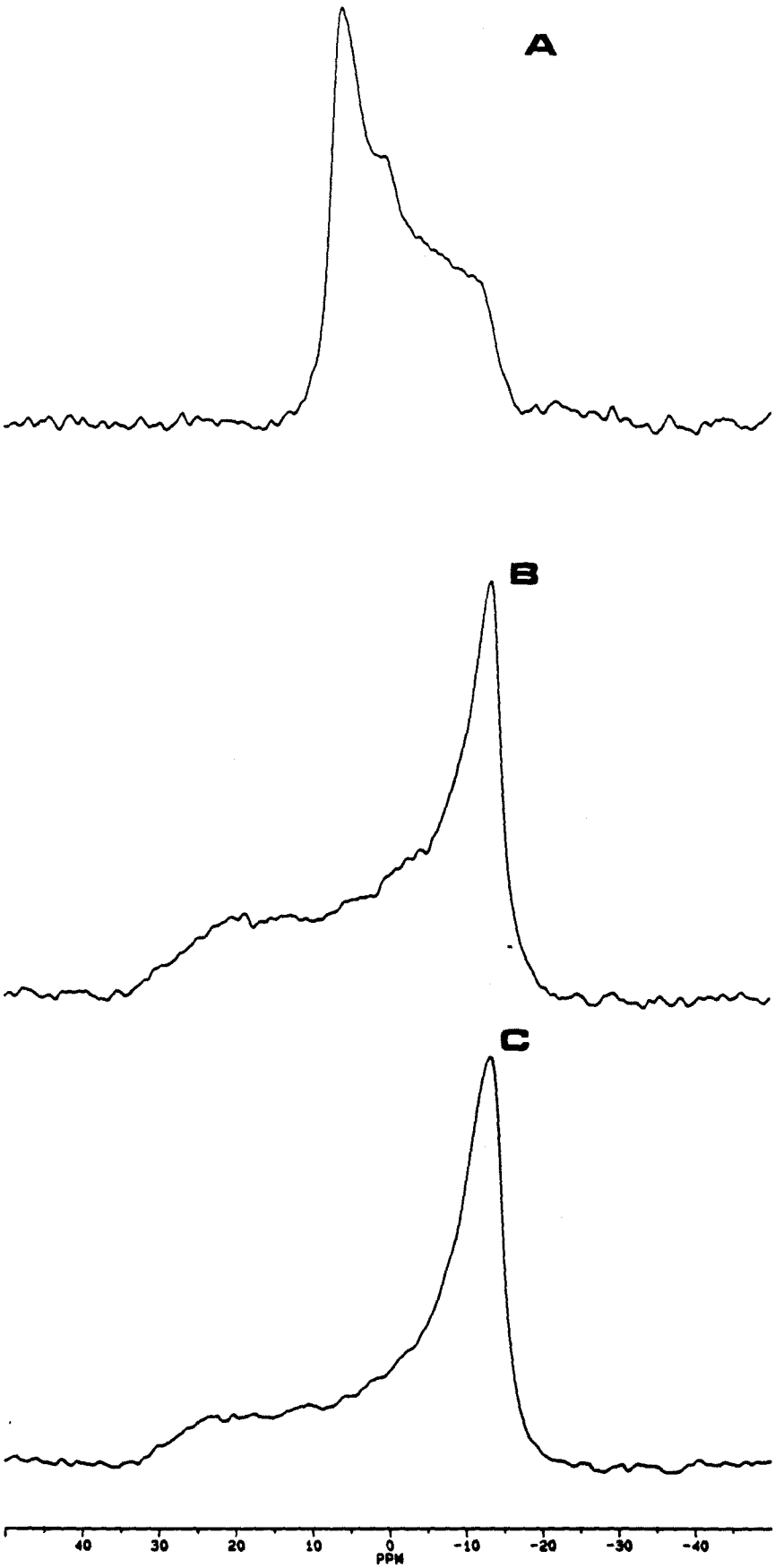

A
B
C
40
30
20
10
0
PPM
-10
-20
-30
-40

Fig. 5. Temperature dependence of ^{31}P-NMR powder patterns for DOPE:TEC (4:1) at (A) 24^{o} C (B) 30^{o} C (C) 35^{o} C.

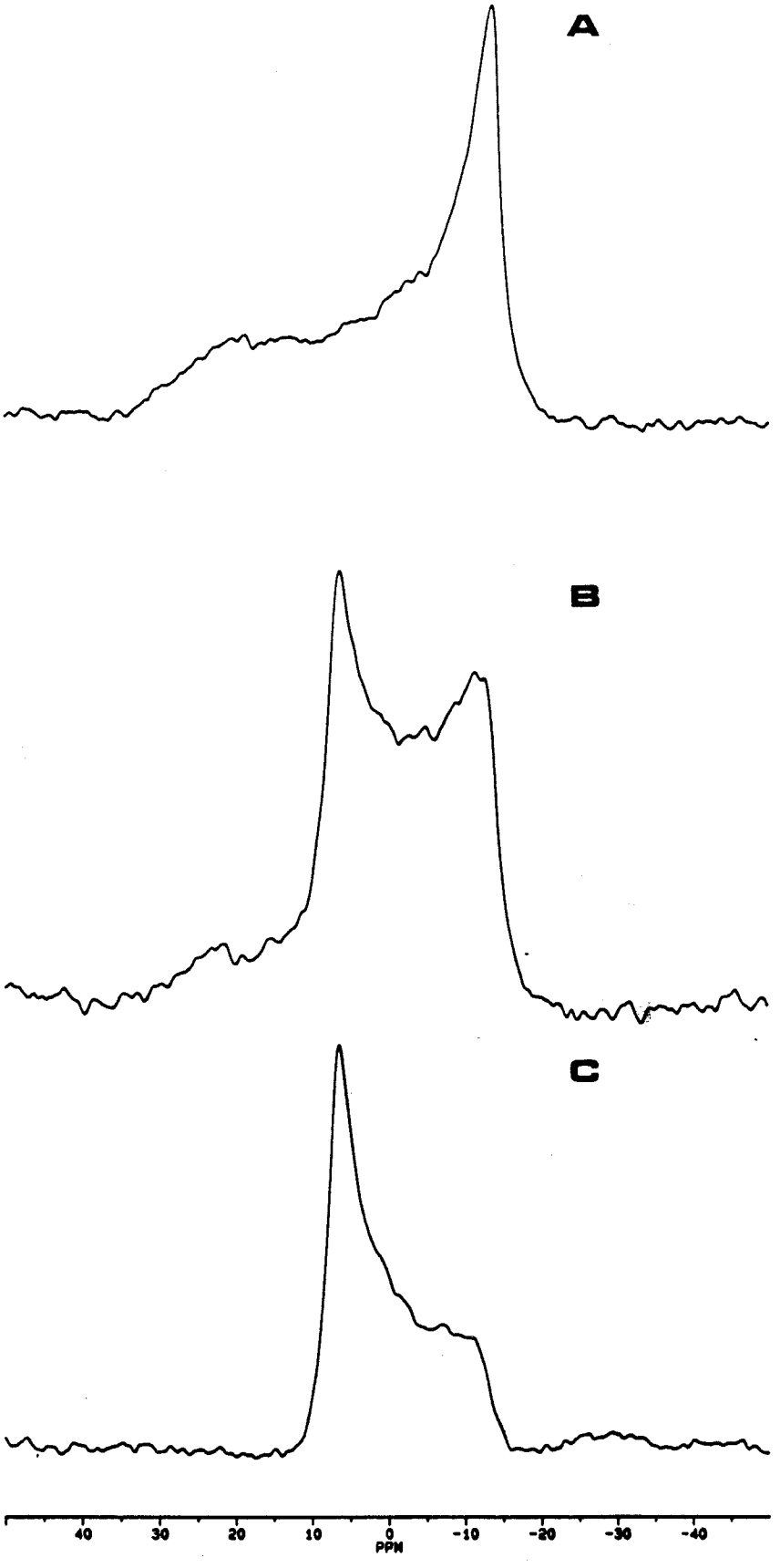
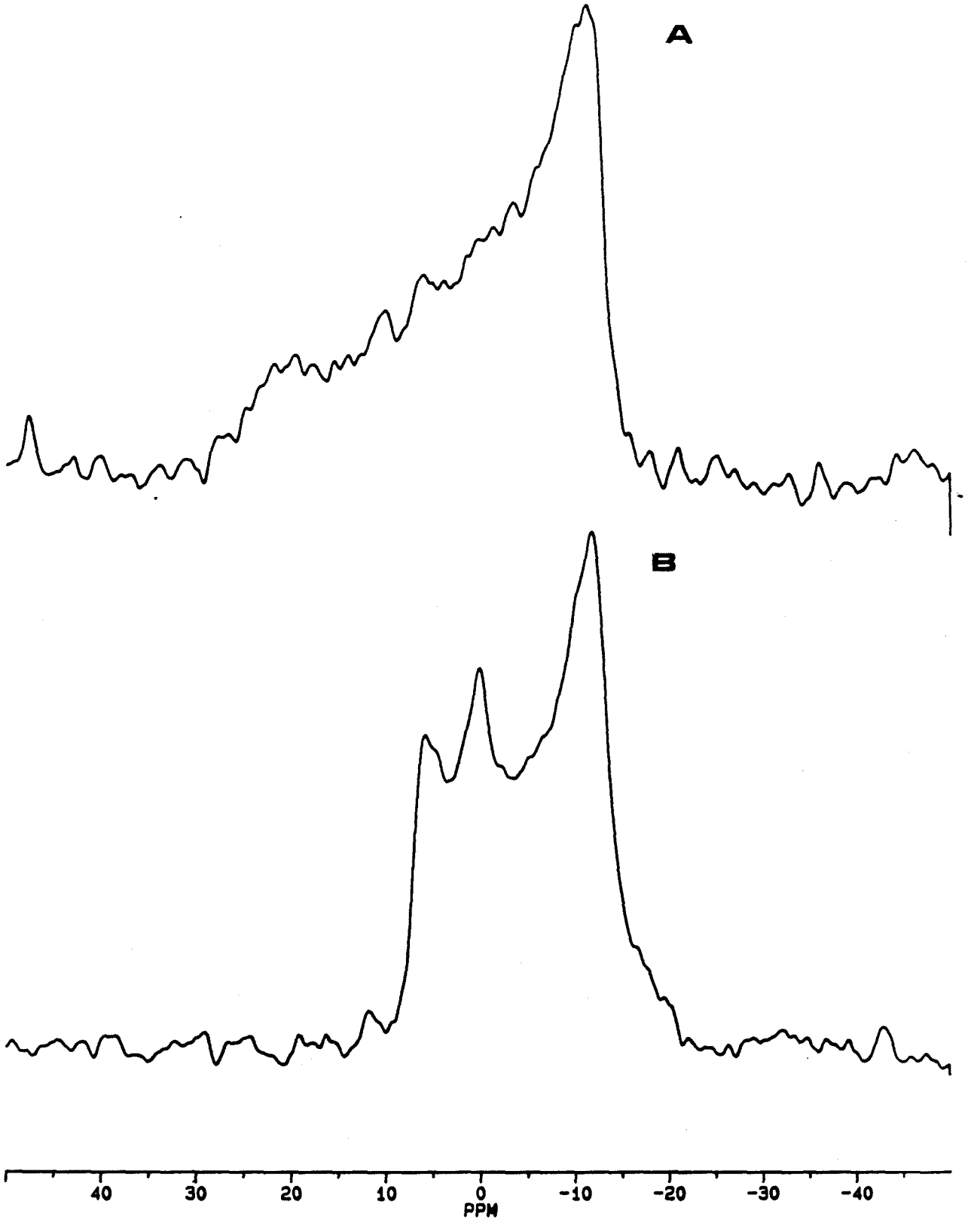

A
B
C
40
30
20
10
0
PPM
-10
-20
-30
-40

Fig. 6. Temperature dependence of powder patterns for DOPE:TEC-Gal (4:1) at (A) 45°C (B) 55° C.


A
B
40
30
20
10
0
-10
-20
-30
-40
PPM

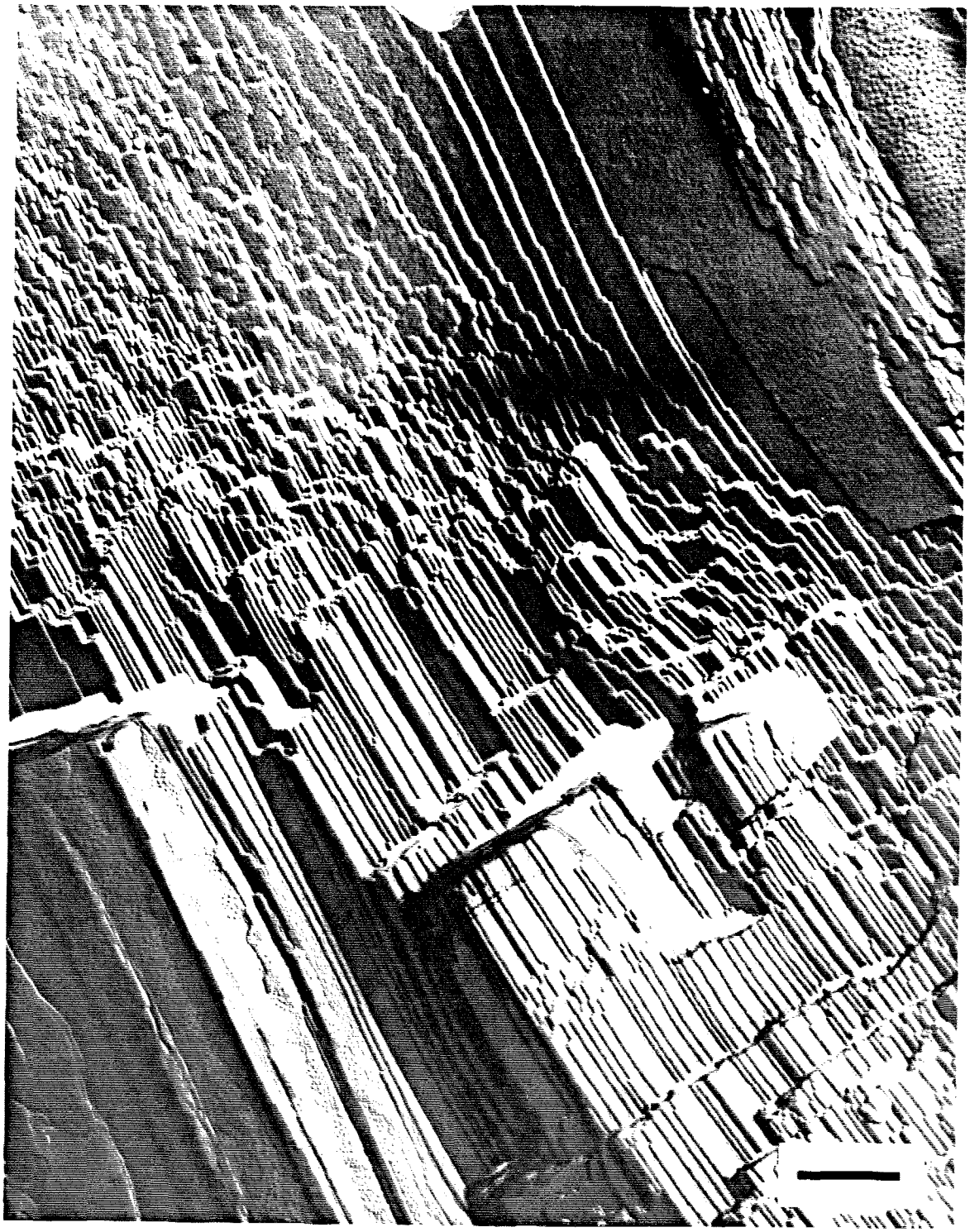
Electron microscopy results

The ^{31}P-NMR results presented above are corroborated by freeze-fracture electron microscopy. Fig. 7 depicts samples prepared from a 9:1 mixture (Top) and a 4:1 mixture (Bottom) of DOPE/TEC-Gal. These samples are characterized by regions with the smooth fracture faces characteristic of the lamellar phase in the form of MLV of 0.2 to 2.0 μm in diameter.[40,41] These results are consistent with those obtained from ^{31}P-NMR, where the presence of lamellar components in the spectra was detected.

Fig. 8 also depicts the freeze-fracture morphology of other regions in the same sample preparations of DOPE/TEC-Gal (9:1) mixtures recorded above. The structures here possess the regular, ribbed appearance characteristic of hexagonal phase arrangement. This is again consistent with the ^{31}P-NMR results that indicated the presence of a hexagonal phase component for this composition. No such structures were detected in samples of DOPE/TEC-Gal (4:1).

Fig. 7. Freeze-fracture electron micrographs of (Top) DOPE:TEC-Gal (9:1) mixture showing presence of lamellar phase (Bottom) DOPE/TEC-Gal (4:1) showing presence of lamellar phase. Bar equals 0.24 μm in all panels.

Fig. 8. Freeze-fracture electron micrography of DOPE:TEC-Gal (9:1). Bar equals 0.24 μm. Sample regions for this composition also possessed characteristics of hexagonal phase arrangement as depicted here.



Discussion:

The results appearing in the previous section demonstrate the capacity of the derivatives to alter the phase behavior of phospholipids. For MLV composed of DPPC, a reduction in the phase transition temperature is observed similar to the 7° C depression in T_m for PC:PS (9:1) SUV upon addition of 1 M trehalose.[4] The implication of this is that the lateral spacing between the molecules in the bilayer is increased. This lowering of the T_m is opposite to the trend reported by Crowe et al. for MLV in the presence of trehalose.[4] These authors indicate that the increase in T_m for the MLV may be a consequence of only a few of the outer lamellae being exposed to the trehalose, resulting in dehydration of the inner lamellae. It is anticipated that equal distribution of the carbohydrate throughout the lamellae would produce a behavior similar to the PC:PS SUV and the DPPC MLV used in this study, since it is incorporated into the lamellae.

In addition to the effects of trehalose on PC, it is also known to inhibit the formation of the hexagonal phase arrangement for DOPE. [11] The bilayer-to-hexagonal phase transition is shifted by as much as 20-30° C above the normal temperature in the presence of trehalose (4 mol/mol lipid). A similar behavior is observed here for the cholesterol derivatives. For TEC-Gal, the lamellar-to-hexagonal phase transition temperature is shifted to a temperature approx. 30° C higher than that for pure DOPE. An increase in the lamellar-to-hexagonal phase transition temperature is also observed for 20 mol% TEC, but the shift is less dramatic.

Several mechanisms may be invoked to explain the results obtained in the case noted above. These include the possibilities that: (i) the derivatives alter the long-range order of water molecules or hydration capacity of the membrane surface. This would result in alterations of phospholipid headgroup orientation and interactions [42]; (ii) some type of coordinate linkage may occur between the derivatives and the phospholipid headgroups such as hydrogen bonding [43]; (iii) the derivatives may interact with the hydrocarbon chains via the steroid portion of the molecule [44]; (iv) steric contraints imposed by the derivatives may alter lipid packing and interactions [45].

All four mechanisms offer tenable explanations for the effects of the derivatives on phosphatidylcholine and phosphatidylethanolamine. For DPPC, the consequence of each of these would be an increase in the lateral spacing of the phospholipids, effectively decreasing the Van der Waals interactions and lowering the T_m as observed.[42,43] For DOPE, an increased spacing and hydration would decrease the potential for hydrogen bonding among the headgroups, which is partially responsible for formation of the hexagonal (H_{II}) phase. Disruption of the intermolecular hydrogen bonding in this manner would promote an entropic driving force for stabilization into the lamellar phase. While present data do not implicate any one of these in the modulation of lipid phase behavior, future studies should elucidate the underlying mechanisms involved.

In summary, the derivatives demonstrate the capacity to lower the gel to liquid-crystalline phase-transition temperature in PC and inhibit the formation of the H_{II} phase for PE. Both appear to be properties of agents used as cryoprotectants. The majority of cryoprotectants studied to date, possess this feature.[12] The effects elicited by the derivatives are more pronounced on a concentration basis than that observed for free carbohydrates. Based on the calculated concentration of the cryoprotectants within a fixed distance from the membrane surface, however, the effects are of the same order of magnitude. This implies that immobilization of a cryoprotectant at the membrane surface is a viable strategy for reducing the amounts of material required to modulate phase behavior. An intriguing question posed by these results is whether or not this approach is equally effective on the property of cryoprotection. The direct structural requirements (such as the presence or absence of carbohydrate, its mobility and position, etc.) needed to produce the cryoprotective action for this system remain to be assessed.

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CHAPTER 2

The Cryoprotective Action of Synthetic Glycolipids

Abstract

Egg PC vesicles frozen and thawed in the presence of carbohydrate derivatives do not incur damage normally associated with freeze thawing. Treated vesicles maintain membrane integrity as evidenced by the lack of lipid intermixing and maintenance of vesicle size following freezing and thawing. This protection is conferred at a derivative:lipid ratio of 0.4 mole/mole, significantly lower than the amount of carbohydrate required when not attached directly to the vesicle. This result indicates that only the carbohydrate at the vesicle surface is responsible for imparting stability to the membrane. This effect can be modulated by variations in the nature of the surrounding medium or alterations in the structure of the carbohydrate, suggesting that direct interactions occur between the carbohydrate and membrane at the membrane interface that are sensitive to bulk phase properties.

Introduction

In the previous chapter, the preparation of a new class of carbohydrate derivatives for use as cryoprotectants was described.[1,2,3] It was also demonstrated that these compounds interacted with phospholipid vesicles as evidenced by their capacity to alter lipid phase behavior in phosphatidylcholine and phosphatidylethanolamine vesicles.[1,2] As noted however, the efficacy of these compounds for use in protecting vesicles against damage induced by freezing and thawing had not been investigated.

Freeze thawing of small unilamellar vesicles results in the formation of larger or multilamellar vesicles with leakage of vesicle contents to the surrounding medium. [4,5] As outlined in Chapter One, this damage is believed to occur via fusion of vesicles or formation of non-lamellar phases such as the H_{II} phase. Specifically, this behavior is induced by the dehydration of membrane surfaces. [6,7] The effects are most pronounced for samples subjected to direct dehydration by sublimation of water, but are also known to be present in samples subjected to freezing.[8] In these cases, water is removed from the vesicle surface due to its crystallization into hexagonal ice. Under such conditions, the membrane is effectively dehydrated. [8] Low water contents and reduced temperatures favor the formation of gel phase as well as nonbilayer phases for several lipid systems. These processes are known to occur in natural as well as model membranes. [6] In natural systems, such alterations often result in lateral phase

separation of membrane components, leakage of contents, and passage through destabilizing phase transitions. Fusion of membranes is one consequence of the fact that such unstable membranes have been produced. By assessing fusion of vesicles, it is thus possible to assess the stabilizing influence of additives on membrane structure under conditions of low water activity.

As noted in Chapter One, it has been demonstrated that the addition of carbohydrates to vesicle suspensions results in retention of vesicle size and integrity following freezing and thawing. [9,10,11] This protective effect is believed to be the result of the interaction of the carbohydrate and the phospholipid component of the membrane. The action of carbohydrates in this regard has been demonstrated exclusively for samples of water soluble carbohydrates added to vesicle suspensions. In these cases, two distinct phases exist: (a) the lipid interfacial phase characterized by a polarity close to that of methanol:water mixtures [12] and (b) the aqueous phase in which the carbohydrate primarily resides. If interactions between the carbohydrate and lipid are to occur in a direct fashion, there must be a distinct interface at the membrane surface at which carbohydrate and lipid co-exist. This implies that only the carbohydrate at the interface is responsible for producing the interactions required for protection and would require that the carbohydrate partition to some extent from the aqueous environment into the less polar lipid interfacial region. Direct incorporation of the carbohydrate into this interface via the derivatives depicted in Fig.1 of the

introduction thus serves as a test of this mechanistic explanation. The rationale for the use of these particular compounds was outlined in Chapter One. In general, if bulk effects are not predominant in determining the stability of the vesicles, then the tethering of the carbohydrate at this interface should result in a protective capacity at a Lipid:Carbohydrate ratio without excess carbohydrate in the bulk solution phase.

The tethering of the carbohydrate at the vesicle surface establishes a fixed distance within which the carbohydrate at the interface can reside. For the derivatives utilized in this study, a sterol backbone was selected to provide the hydrophobic portion of the molecule capable of residing in the acyl chain region of the vesicle bilayer. The position of the sterol in the bilayer was confirmed by the behavior elicited by these compounds when incorporated into DPPC and DOPC vesicles.[1] For this study, with derivatives where n=3, and the repeat distance along the chain axis is 1.9-3.5 Angstroms, the maximum extension of the carbohydrate away from the bilayer surface can be estimated at between 5.7 and 10 Angstroms. [13,14] Since the polar group of phospholipids is known to extend 10-12 Angstroms from the plane of the bilayer, this places the carbohydrate group in the lipid headgroup region at the lipid/water interface. [15]

The specific carbohydrates utilized in this study were galactose and maltose. Each serves as a representative of a monosaccharide and disaccharide respectively. No distinct differences have been demonstrated to exist in the past when

mono- and disaccharides have been compared in terms of efficacy in freeze/thaw protection.[16] The use of both types of compounds in this study further tests whether or not differences in behavior may be elicited due to alterations in the extent of interaction at the bilayer interface. The compound in which the terminal -OH group on the polyoxyethylene linker is underivatized possesses all functionalities except the carbohydrate and thus represents an appropriate control for assessing the role of the carbohydrate moiety in eliciting the cryoprotective action.

Materials and Methods

To test the effectiveness of the compounds used in this study for providing stabilization during freezing, their capacity at preventing aggregation and fusion was assessed via light scattering and a resonance energy transfer assay. As noted above, when vesicles are subjected to freezing and thawing, alterations in membrane structure occur that lead to fusion of membranes. The result of such behavior is intermixing of membrane surfaces and formation of larger vesicle structures such as multilamellar vesicles. [16] This behavior in biological systems is incompatible with viability. By examining the capacity of the carbohydrates tethered to the vesicle surface for preventing these phenomena, one can gain a relative measure of the effectiveness of the interactions involved in their cryoprotective action.

Egg phosphatidylcholine was obtained from Avanti Polar Lipids and used without further purification. The derivatives were prepared according to the procedures described previously.[1] Vesicles were prepared by drying a stock solution of lipid from chloroform overnight under mechanical pump vacuum. The resulting lipid film was subsequently hydrated with a buffer containing 120 mM sodium chloride, 10 mM Hepes, pH 7.4. The suspension was then vortexed briefly, followed by sonication for 20-30 minutes in a bath sonicator from Laboratory Supplies of Hicksville, New York. Large vesicles and multilamellar vesicles

were removed by centrifugation at 12,000 x g for 5 minutes. All vesicle suspensions were prepared at a final lipid concentration of 14 mg/ml.

For freezing and thawing, samples were immersed in a container of liquid nitrogen for 3 hours. Samples were thawed at room temperature and allowed to stand for several hours before further analysis was performed.

Resonance Energy Transfer Assay

For the resonance energy transfer assay, lipids were sonicated with the addition of the appropriate fluorescent probe. The fluorescent probes comprised 1 mole % of total lipid. A donor probe of N-(7-nitro-2-1,3-benzoxadiazol-4-yl) dipalmitoyl phosphatidylethanolamine and an acceptor probe of N-(lissamine rhodamine B sulfonyl) dioleoyl phosphatidylethanolamine were used in this study. The donor (NBD) and acceptor (RHO) vesicles were combined in a 1:3 (NBD:RHO) ratio. By using this ratio of donor:acceptor, even slight levels of intermixing result in noticeable levels of donor emission quenching. For freezing, a sample of 200µl of the mixed vesicles was frozen in liquid nitrogen and allowed to warm to room temperature. Sample temperature was followed using a thermocouple placed in the sample tube. Samples were allowed to stand for one hour at room temperature before analyses were performed. Spectra were

recorded using an SLM-4800 spectrofluorometer with excitation at 450 nm and emission monitored from 470-620 nm. Mock fused vesicles bearing both donor and acceptor probes in a 1:1 ratio were used as the 100% mixing standard. A calibration chart was prepared for each sample set using various proportions of untreated mixed and mock fused vesicles. Donor (NBD) emission at 530 nm was used to determine the extent of probe mixing. [17]

Light Scattering Measurements

Vesicles were assayed to determine vesicle size before and after being subjected to the freeze thawing cycle. As noted above, freeze thawing of Egg PC vesicles results in the formation of large multilamellar vesicles. [16] As a consequence, there is a pronounced increase in the vesicle size. Light scattering was recorded using the Malvern 4700C sub-micron particle analyzer. A Spectraphysics series 2000 Argon-ion laser operating at 488 nm was used as an excitation source. The intensity of the scattered light was recorded with a photomultiplier tube oriented at a 90 degree angle to the excitation beam. The resulting intensity distribution was analyzed to obtain vesicle size distribution by number.

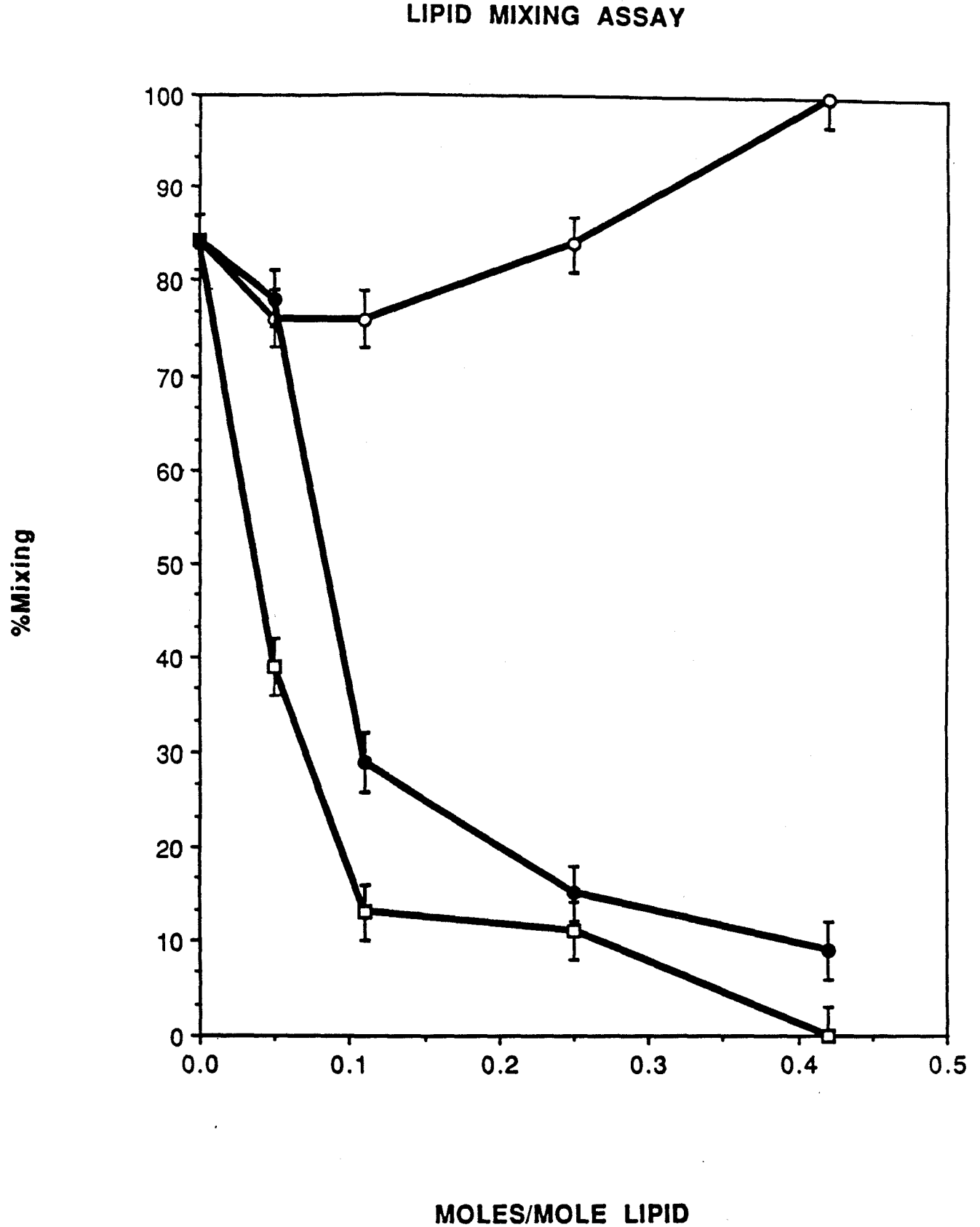
Results and Discussion

Resonance Energy Transfer

Fig. 1 displays the results of the resonance energy transfer assay for Egg PC vesicles that had been subjected to freeze thawing. High levels of intermixing are known to correspond to high levels of fusion in vesicle samples. [5] Increasing amounts of TEC result in enhanced levels of probe intermixing. This behavior is distinct from the case in which TEC-Gal or TEC-Mal are added to the vesicles. In these cases, increasing amounts of either derivative result in reduced levels of probe intermixing. This effect appears to be slightly more pronounced for the disaccharide derivative with a minimum level of mixing occuring between ratios of 4:1 and 7:3 moles of lipid/mole derivative. Since the maximum effect was observed in this range, a 7:3 ratio of Lipid:Carbohydrate derivative was utilized in all subsequent experiments.

The incorporation of the carbohydrate into the membrane requires that a stable complex be formed in which the sterol portion of the molecule is intercalated directly into the membrane. This complex should not be dissociated upon dilution of the vesicle suspension. Under normal conditions, with the carbohydrate existing in the aqueous phase, dilution of the lipids results in a decreased likelihood of vesicle/carbohydrate interaction. This in turn results in a decreased capacity of the vesicles to withstand freeze thawing. [11] What is required is that a distinct

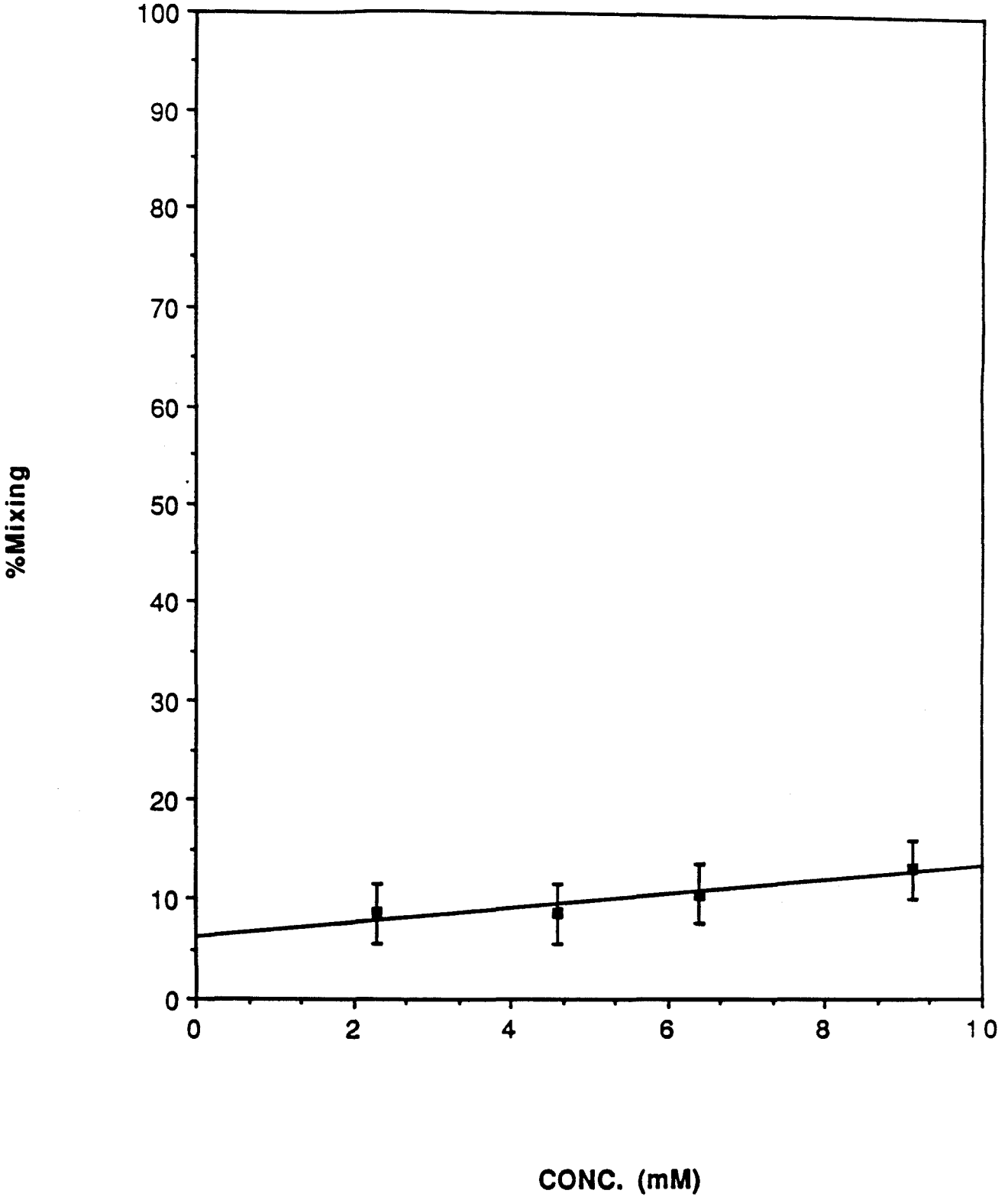
Fig. 1. Resonance energy transfer assay as a function of the concentration of derivatives. All sample preparations were maintained at 14 mg lipid/ml. Vesicles containing NBD were mixed with vesicles containing RHO in a 1:3 ratio prior to freezing and thawing. The samples were frozen in liquid nitrogen for 3 hours and subsequently allowed to warm to room temperature. After one hour at room temperature, analyses were performed using 450 nm excitation and scanning emission from 470 to 620 nm. The extent of donor (NBD) quenching at 530 nm was used as a measure of the amount of intermixing. Percent mixing was taken from a calibration chart constructed by mixing the mixed vesicles prior to freeze thawing (0% mixing) with vesicles containing both probes in the same vesicles (100% mixing). O TEC ● TEC-Gal ■ TEC-Mal.


LIPID MIXING ASSAY
%Mixing
100
90
80
70
60
50
40
30
20
10
0
0.0
0.1
0.2
0.3
0.4
0.5
MOLES/MOLE LIPID

stoichiometry be maintained at the surface which is not altered by variations in carbohydrate concentrations at the interface or variations in the amount of lipid present. The results of the dilution experiment are depicted in Fig. 2. A sample of Egg PC:TEC-Mal (7:3) was prepared and diluted to several final lipid concentrations. These samples were then frozen in liquid nitrogen and thawed. The level of intermixing was then plotted versus lipid concentration. As depicted in Fig. 2, the dilution of this vesicle suspension results in *decreased* levels of intermixing . This behavior is consistent with a decreasing potential for vesicle fusion due to a decreased likelihood for intervesicular contact and suggests that a stable complex is indeed formed that is insensitive to dilution effects. In each case, though the lipid concentration has been decreased, the relative concentration of the carbohydrate at the membrane surface has remained the same. The stoichiometry for the lipid and carbohydrate complex is not changed.

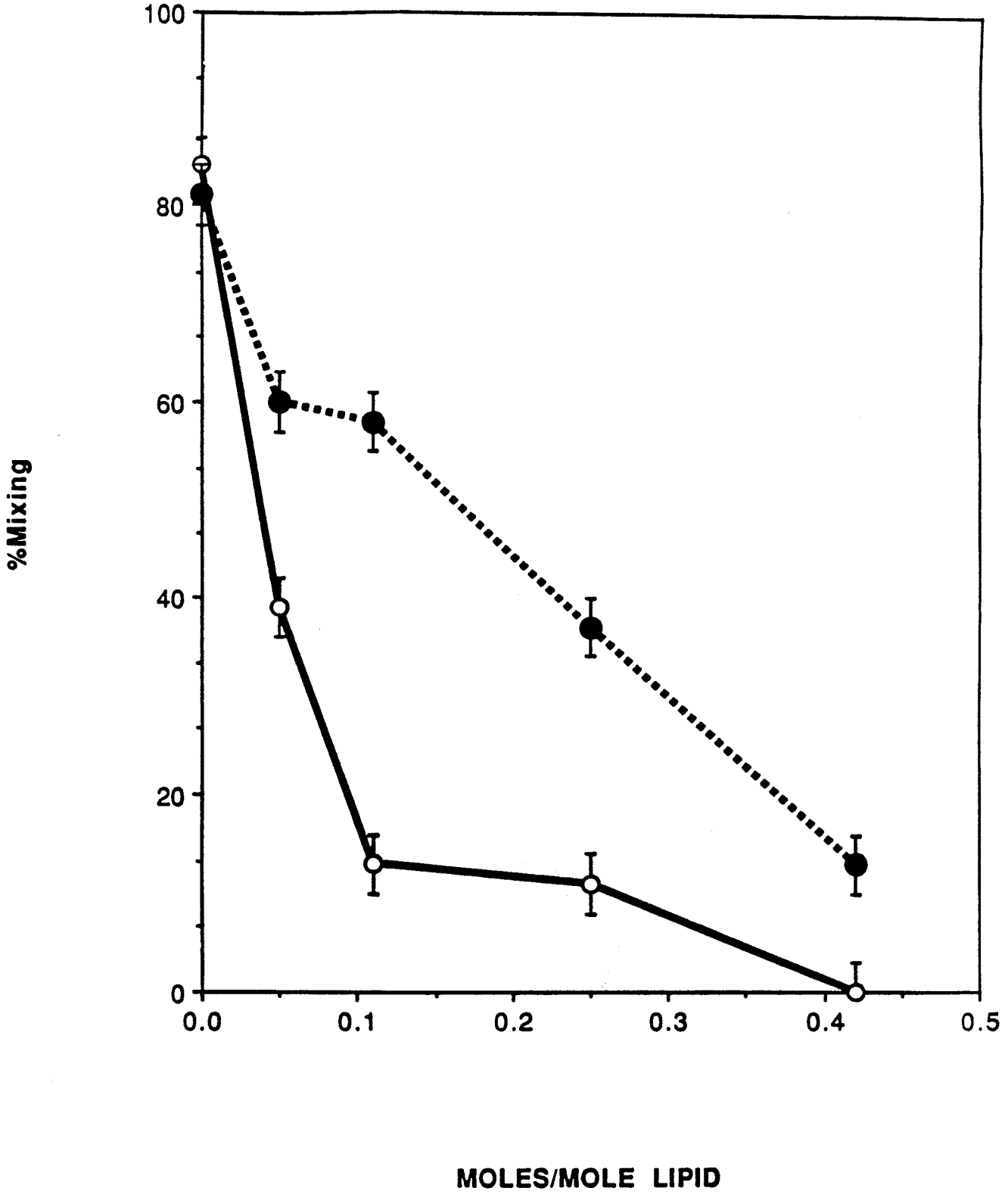
Results of several other workers have suggested that the extent of the carbohydrate interaction with the vesicle bilayer can also be moderated by the addition of a variety of agents to the vesicle suspension, that modify or block the interaction of the compounds with the lipids in the bilayer. [17] The problem with such studies, however, arises from the fact that the addition of these compounds, usually in the form of salts, to the aqueous phase also alters the structure of the surrounding water matrix. Such effects might also be responsible for the observed amelioration in the

Fig. 2. Lipid mixing as a function of concentration of Lipid. The line generated can be fit by the equation $y=6.2 + 0.7x$ with a correlation coefficient of 0.94.


100
90
80
70
60
50
40
30
20
10
0
%Mixing
0
2
4
6
8
10
CONC. (mM)

carbohydrate effect because they have also been demonstrated to inhibit the partitioning behavior of carbohydrates into interfacial phases.[18] To test the effects induced by alterations in the surrounding medium without the addition of chaotropic agents to the suspensions, vesicle preparations were made substituting D_2O in place of H_2O in the buffers. The use of D_2O alters the environment in several ways without altering or blocking specific sites for interaction. In particular, since the -OD group forms stronger hydrogen bonds than the -OH group, the use of D_2O favors an increase in the structure of the surrounding matrix. This is represented primarily by a higher degree of hydrogen bonding in the surrounding matrix. [19] The results depicted in Fig. 3 demonstrate a pronounced capacity for intermixing for samples produced in D_2O. The use of D_2O for samples lacking the TEC-Mal does not produce any discernible difference in the level of intermixing. For samples containing TEC-Mal at various ratios, however, there is a distinct increase in the amount of intermixing when comparing samples in D_2O versus H_2O. This result indicates that the use of deuterium in place of hydrogen alters the interaction responsible for the cryoprotective action exerted by the carbohydrates. In particular, this further suggests that the medium surrounding the carbohydrate at the lipid interface is of critical importance in determining the effectiveness of the cryoprotective action.

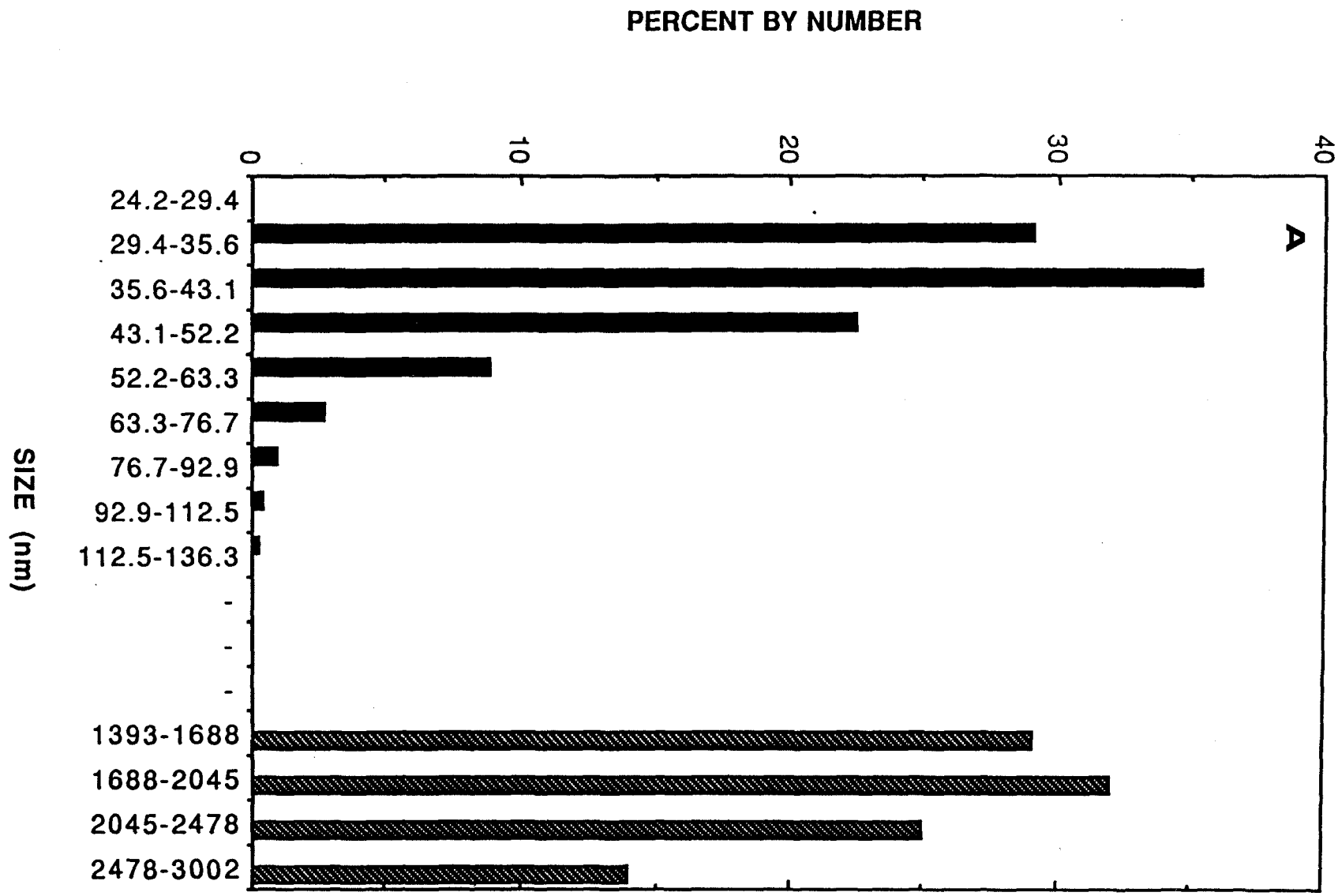
Fig. 3. Lipid mixing in D_2O versus H_2O. Lipid concentration in all samples was maintained at 14 mg/ml ● D_2O O H_2O.

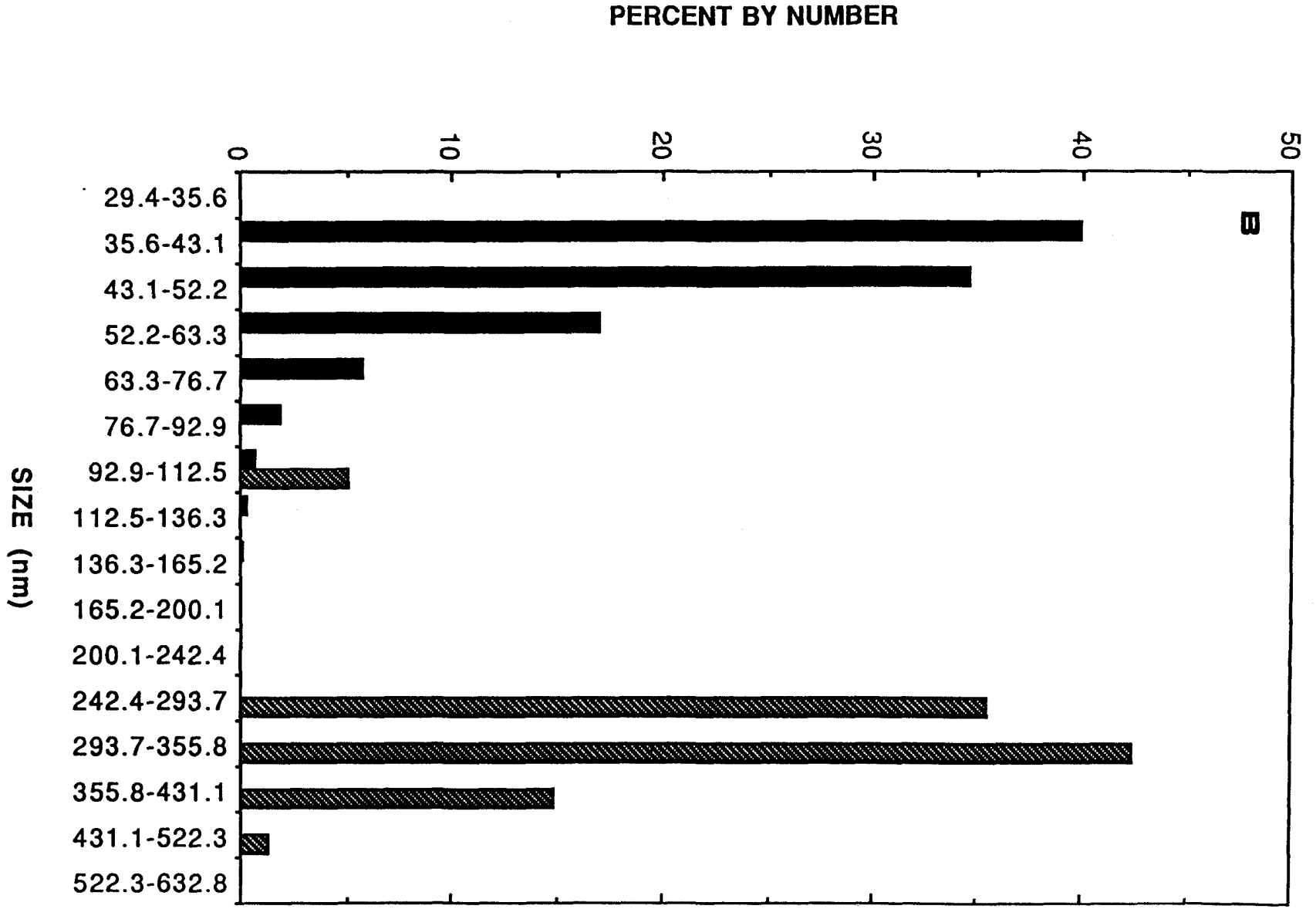

100
80
60
40
20
0
0.0
0.1
0.2
0.3
0.4
0.5
%Mixing
MOLES/MOLE LIPID

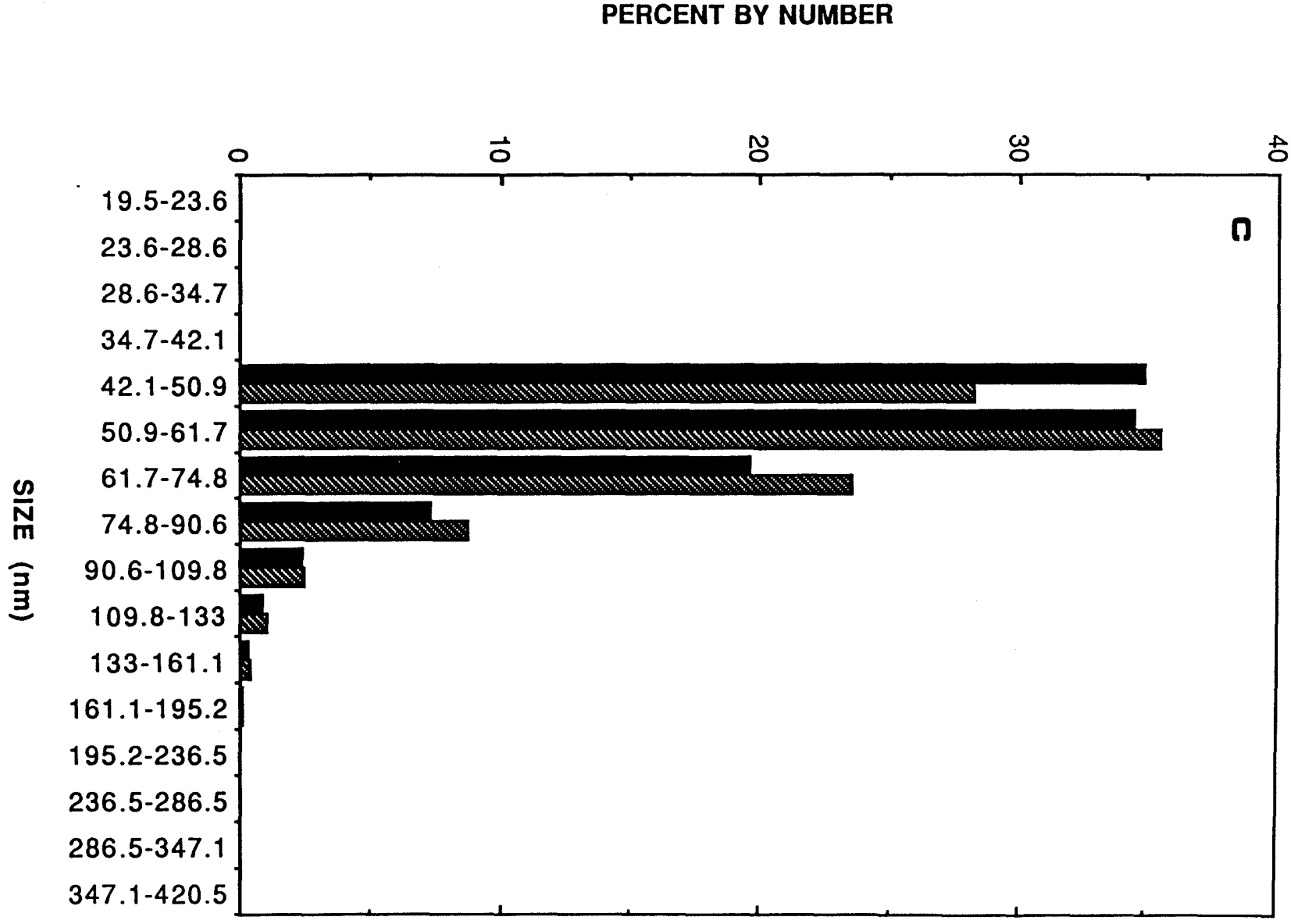
Light Scattering Measurements

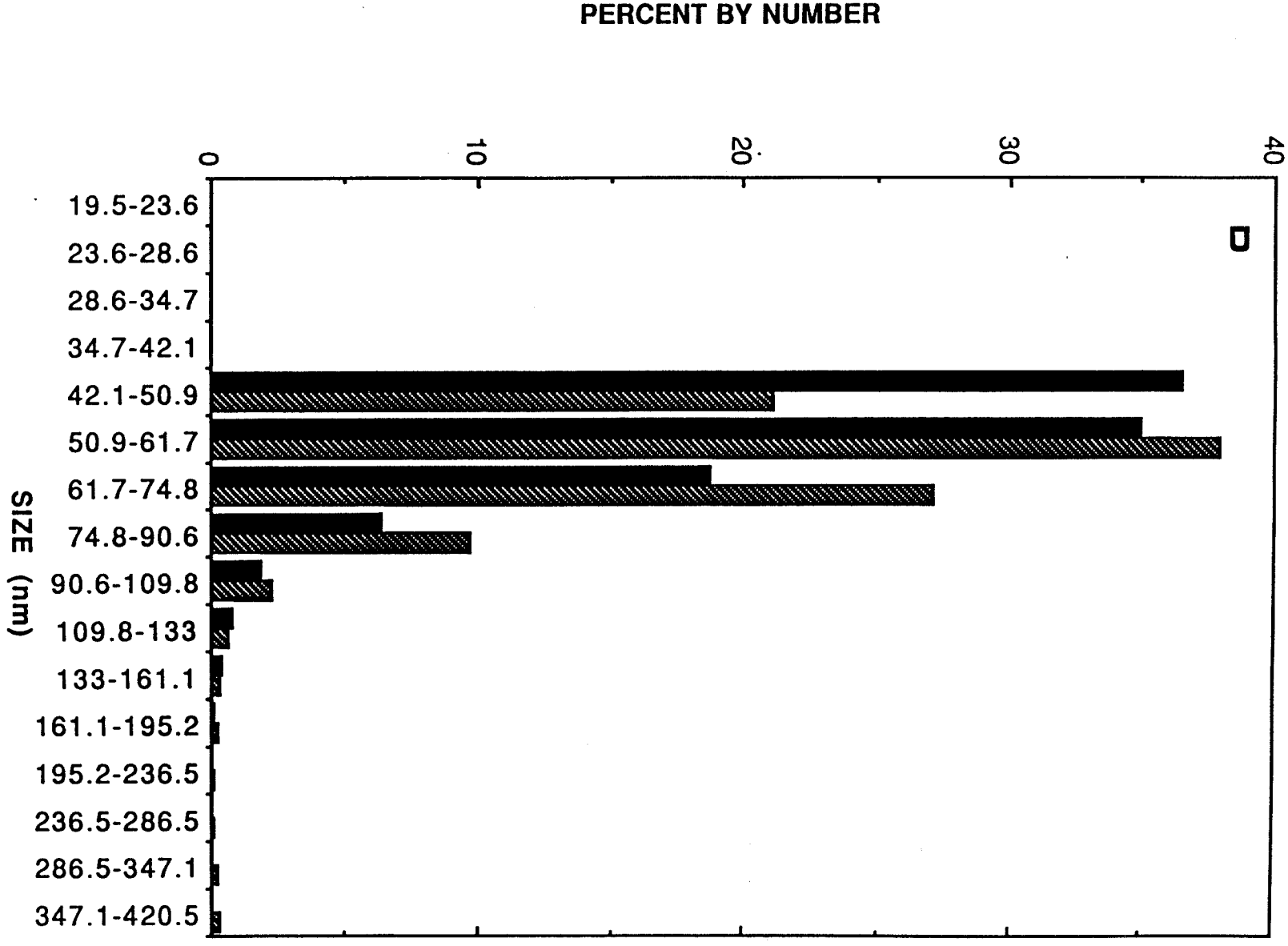
Fig. 4 depicts light scattering results obtained for samples before and after freeze thawing. Egg PC vesicles frozen and thawed without additive form vesicles in the size range of 1-2 microns. These vesicles start out with an average distribution by number at 42.5 +/- 0.1 nm. This is also true of vesicles prepared in the presence of TEC (Egg PC:TEC (7:3)). In this case, vesicles of 48.9 +/- 0.6 nm show an increase in size to 242.1 +/- 116.8 nm. The addition of TEC-Gal or TEC-Mal (lipid:carbohydrate (7:3)) , however, yields quite distinct results. In these cases, the vesicles following freezing and thawing show only slight increases in the recorded vesicle sizes. Vesicles with TEC-Mal for example start out at an average size of approximately 58.7+/- 1.1 nm and increase in size following freezing and thawing to 64.0 +/-3.1 nm. The results obtained in this case are consistent with those recorded using the resonance energy transfer assay. Notably, the addition of carbohydrates to the bilayer results in maintenance of membrane integrity. Fusion or aggregation of vesicles would normally result in the formation of larger particles and intermixed membranes. These phenomena have been observed only in cases for samples in which carbohydrate was not present.

Fig. 4. Vesicle size before and after freeze thawing recorded as distribution by number of particles in the given size ranges. (A) Egg PC, (B) Egg PC:TEC (7:3), (C) Egg PC:TEC-Gal (7:3), (D) Egg PC:TEC-Mal (7:3). In all cases, sizes before freezing and thawing are depicted in solid black ■ Those recorded following freezing and thawing are recorded with ▨.


PERCENT BY NUMBER
0
10
20
30
40
A
SIZE (nm)
24.2-29.4
29.4-35.6
35.6-43.1
43.1-52.2
52.2-63.3
63.3-76.7
76.7-92.9
92.9-112.5
112.5-136.3
1393-1688
1688-2045
2045-2478
2478-3002


PERCENT BY NUMBER
0
10
20
30
40
50
SIZE (nm)
29.4-35.6
35.6-43.1
43.1-52.2
52.2-63.3
63.3-76.7
76.7-92.9
92.9-112.5
112.5-136.3
136.3-165.2
165.2-200.1
200.1-242.4
242.4-293.7
293.7-355.8
355.8-431.1
431.1-522.3
522.3-632.8
B


PERCENT BY NUMBER
0
10
20
30
40
C
SIZE (nm)
19.5-23.6
23.6-28.6
28.6-34.7
34.7-42.1
42.1-50.9
50.9-61.7
61.7-74.8
74.8-90.6
90.6-109.8
109.8-133
133-161.1
161.1-195.2
195.2-236.5
236.5-286.5
286.5-347.1
347.1-420.5


PERCENT BY NUMBER
0
10
20
30
40
D
SIZE (nm)
19.5-23.6
23.6-28.6
28.6-34.7
34.7-42.1
42.1-50.9
50.9-61.7
61.7-74.8
74.8-90.6
90.6-109.8
109.8-133
133-161.1
161.1-195.2
195.2-236.5
236.5-286.5
286.5-347.1
347.1-420.5

Conclusions

The results recorded here indicate that carbohydrates directly attached to the membrane via an anchoring group are capable of exerting the same effects noted for free carbohydrates added to a vesicle suspension. The amount of derivative required to induce the cryoprotective capacity is on the order of *100 times lower* than that recorded for the amount of trehalose and sucrose required to reduce probe intermixing to comparable levels (40:1 Carbohydrate:Lipid molar ratios). [10,11,17] The addition of untethered carbohydrates to vesicle suspensions results in two distinct environments for the carbohydrates. There is in one instance, the hydrophilic aqueous environment and in the other instance, the less hydrophilic lipid interfacial region. If the carbohydrate interaction with the membranes is a direct consequence of interaction with the lipids in the membrane, then attachment of the carbohydrate to the membrane should enhance this effect by increasing the relative concentration at the interface. The excess carbohydrate in the external aqueous environment is not required if this mechanism is valid. The results recorded here suggest that this is indeed the case, and further suggest a defined stoichiometry of carbohydrate required at the membrane interface in order to induce this effect. The effects noted here are maximized for instance at Carbohydrate:Lipid molar ratios of 0.4:1.

The differences in the cryoprotective capacity of the derivatives in D_2O versus H_2O indicate that the interaction of the carbohydrate at the interface is governed in part by the external medium in which the vesicle formulation is placed. Alterations in the medium could, for example, alter the partitioning behavior of the carbohydrate from the aqueous environment into the less hydrophilic environment at the lipid interfacial region.[20] This would then lead to a decreased opportunity for interactions at the interface. In this manner, the nature of the medium could ameliorate the cryoprotective capacity of the carbohydrates. Partitioning behavior of solutes is dependent on several factors including entropic factors. [20,21] These effects have been demonstrated previously for carbohydrates partitioning into butanol and polystyrene beads from an aqueous phase.[18] Alterations in the order of the system could alter the partitioning of the carbohydrate into the less hydrophilic interfacial region and thus modify its interactions with membrane lipids. This behavior suggests a possible correlation between the capacity of the carbohydrates to intercalate into the interface and the capacity to exert a cryoprotective action.

The behavior observed for the derivatives also indicates that the cryoprotective capacity is a consequence of having the carbohydrate at the interface. The derivative designated as TEC possesses all of the functionalities of the other compounds except the carbohydrate. This compound does not demonstrate a capacity to prevent fusion in vesicles subjected to freezing and thawing.

There do not appear to be any significant differences,however, in samples containing galactose or maltose as the terminal sugar. This result suggests that cryoprotection is a characteristic of saccharides in general, i.e., not specifically mono- or disaccharides, and further indicates that attachment to the vesicle bilayer does not alter this capacity, but rather enhances the effect.

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CHAPTER 3

Protection of Vesicles Against Damage During Freeze Drying by Addition of Membrane Associated Carbohydrate Derivatives

Abstract

Vesicles of Egg PC or POPC subjected to freeze drying in the presence of several carbohydrate derivatives were protected against damage normally associated with dessication. This protection was evident in the capacity of vesicles bearing the carbohydrates to retain the aqueous marker carboxyfluorescein, maintain membrane integrity without lipid intermixing, and retain the same size following dehydration and rehydration. This protection is conferred by means of incorporation of compounds bearing carbohydrate head groups into the phospholipid vesicles. Protection is evident at stoichiometries of as little as 0.5 moles Carbohydrate/mole Lipid, a reduction from that normally observed for free carbohydrates added to vesicle suspensions. The extent of protection conferred is dependent upon the structure of the carbohydrate used, a disaccharide derivative of maltose exhibiting better protection than a monosaccharide derivative of galactose. The presence of a negatively charged compound in the form of L-(+)-Ascorbic acid also significantly enhances the protective action of the carbohydrate. These results suggest that it is alterations in the membrane surface induced by the presence of carbohydrate at the interface that creates the capacity to survive dehydration, and not alterations in the medium induced by the presence of an excess phase of carbohydrate. The results also further suggest that the extent of interaction at the interface is dependent on the carbohydrate present at the interface and the nature of the surrounding medium prior to freeze drying.

Introduction

The results presented in Chapter Two indicate that the freeze-induced damage to membranes is ameliorated by the addition of membrane-bound carbohydrates. The damage normally elicited in this case is a direct consequence of the dehydration occurring upon freezing of membrane associated H_2O into hexagonal phase ice. A more direct and complete means of dehydration of the membrane surface occurs as a consequence of sublimation or evaporation of water. Even under these conditions, however, carbohydrates can afford protection to membranes. As Crowe and Crowe have demonstrated, this protection under the more severe dehydration conditions is more dependent on the structure of the carbohydrate and nature of the membrane under study.[1,2,3]

In this chapter, the capacity of tethered carbohydrates to protect membranes against direct dessication induced damage is examined. For this purpose, vesicles composed of Egg PC or POPC were utilized. Small unilamellar vesicles (SUVs) of these lipids normally form large multilamellar vesicles (MLVs) upon freezing and drying without additives. This results in a clear indication of fusion and aggregation, two major sources of damage in these systems. The lipids used in this study represent a naturally occurring lipid in the form of Egg PC and a synthetic analog in the form of POPC. The liposomes that are formed from these lipids are also in a liquid-crystalline phase at room temperature and pH 7.4, a condition that corresponds to a normal physiological state.

Several liposome formulations have been utilized in the past for studies involving freeze-drying or freeze thawing. These include POPC:PS (9:1) formulations, Egg PC, and Egg PC:PA (7:3). [4,5,6] In each of the cases involving freeze drying studies, negative charge lipids in the form of PS or PA have been included in the formulations. Recently, Crowe et al. have demonstrated a direct dependence on freeze drying stability and the presence of the negative charged lipid. [7] In order to examine this phenomena further, we also utilized compounds bearing negative charge for protecting vesicles against damage.

Materials and Methods

Egg PC and POPC were obtained from Avanti Polar Lipids of Birmingham, Alabama and were used without further purification. TES, EDTA, and L-(+)-Ascorbic acid were obtained from Sigma Chemical company. Carboxyfluorescein was obtained from Kodak chemicals and was purified prior to use by the method described by Weinstein et al.[8] All buffers containing ascorbic acid were stored in the dark at -80^{o} C under nitrogen to avoid decomposition. All solutions were degassed to avoid oxidation of the ascorbic acid. Sephadex G-25 and LH-20 were obtained from Pharmacia. The derivatives depicted in Fig. 1 of the introduction were synthesized and purified according to the procedures outlined previously. [9]

Vesicles were prepared by drying the appropriate stock solutions of compounds in chloroform under nitrogen and subsequently under vacuum for 24 hours to remove trace organic solvent. The lipid film was then hydrated using the appropriate buffer. The suspension was vortexed briefly and subsequently bath sonicated for 20-30 minutes in a sonicator from Laboratory Supplies of Hicksville, New York. The clear vesicle suspension was then centrifuged at 14,000 x g for 5 minutes to remove large vesicles or multilamellar vesicles. The resulting suspension was then treated according to the appropriate protocol described below. For freeze drying, samples were placed in 3 mls borosilicate glass tubes and immersed in liquid nitrogen while swirling. The samples were frozen in this manner and subsequently transferred to a Labconco model 4.5 benchtop lyophilizer. Samples were dried for 24-48 hours under a vacuum of less than 100 mtorr. All samples were rehydrated with distilled water at room temperature and allowed to stand for one hour prior to performing subsequent analyses.

Light Scattering Measurements

Samples were prepared in TES buffer containing 10 mM TES, 50 mM L-(+)-Ascorbic acid, and 0.1 mM EDTA at pH 7.2. Lipid concentration was maintained at 17 mM. Samples of 200 µl were subjected to freeze drying and rehydrated as described above. Each was examined before and after freeze drying using laser light scattering. A Malvern 4700c sub-micron particle analyzer was utilized. Excitation was carried out around 488 nm using a Spectraphysics argon-ion laser. Light scattering was measured at a 90 degree angle to the excitation beam using a photomultiplier tube. The scattering intensity profile was analyzed to provide a distribution of vesicle size by number.

Carboxyfluorescein Release and Retention

For measuring carboxyfluorescein release, vesicles were prepared in TES buffer containing 10 mM TES, 0.1 mM EDTA, 50 mM L-(+)-Ascorbic acid, and 100 mM Carboxyfluorescein. Unentrapped carboxyfluorescein was removed by passing vesicles over a Sephadex G-25 column. Vesicles were removed in the void volume. Ascorbic acid was added back to the samples following passage over the column to provide an appropriate final external concentration. Following freeze drying and rehydration of 150 µl aliquots, vesicles were examined for extent of leakage using an SLM-4800 spectrofluorometer. Excitation was carried out at 492 nm and emission was monitored at 520 nm. The 0% lysis value was obtained using vesicles not subjected to freeze drying. The 100% value was obtained by lysing the vesicles through addition of Triton X-100 (final concentration 1% by weight). The following formula was then applied to calculate % Retention:

$$\% \text{ Retention} = \frac{(F_a'/F_b')-(F_b'/F_a')}{(F_a/F_b)-(F_b/F_a)} \times 100$$

F_a= Intensity before freeze drying, after triton addition

F_b=Intensity before freeze drying, before triton addition

F_a'=Intensity after freeze drying, after triton addition

F_b'=Intensity after freeze drying, before triton addition

Resonance Energy Transfer Assay

For samples used in the resonance energy transfer assay, vesicles were prepared with donor and acceptor fluorescent probes. A donor probe of N-(7-nitro-2-1,3-benzoxadiazol-4-yl) dipalmitoyl phosphatidylethanolamine (NBD) and an acceptor probe of N-(lissamine rhodamine B sulfonyl) dioleoyl phosphatidylethanolamine (RHO) were used in this study. The fluorescent probes comprised 1 mole% of the total amount of lipid present. Donor and acceptor vesicles were combined in a 1:1 ratio. Samples were prepared in buffer containing 10 mM TES, 0.1 mM EDTA, and 50 mM L-(+)-Ascorbic acid at pH 7.2. Samples of 200 μl of the mixed vesicles were freeze dried at lipid concentrations of 13 mM according to the protocol described above. Following rehydration, samples were examined using the SLM-4800 spectroflourometer with excitation at 450 nm and emission monitored from 470-620 nm. Mock fused vesicles containing a 1:1 ratio of NBD:RHO were used to determine the 100% mixing value. A calibration curve was constructed using various proportions of untreated mixed and mock fused vesicles. Donor (NBD) emission at 530 nm was used to determine the extent of probe mixing.

Transfer Assays

For examining the extent of transfer of TEC-Mal into pure Egg PC vesicles, SUV's of pure Egg PC were prepared in the presence of carboxyfluorescein as described above at a final lipid concentration of 13 mM. To a suspension of these vesicles was added a hand shaken dispersion of TEC-Mal at a concentration of 13 mM. It has been demonstrated that such a dispersion of TEC-Mal consists of MLV's. [11] The vesicles were incubated in the TEC-Mal suspension at various temperatures for varying lengths of time. Following the incubation, the suspension was centrifuged at 12,000 x g for 5 minutes. The supernatant was removed and subsequently passed over a Sephadex G-25 column. Aliquots of 150µl of the eluant were freeze dried as above and analyzed for carboxyflourescein leakage. In addition, the vesicle suspension was extracted using chloroform:methanol 4:1. The organic layer was removed and dried over anhydrous calcium sulfate. The solvent was subsequently removed by evaporation under nitrogen and under vacuum. Thin layer chromatography was performed on the residue using silica gel plates and a chloroform:methanol (4:1) eluant. Spots on the plate were visualized using sulfuric acid. Colors of each spot were recorded with the corresponding R_f value and compared to control lanes of pure TEC-Mal and Egg PC.

Results and Discussions

Freeze drying of vesicles composed of pure Egg PC or POPC with various amounts of the additives present resulted in fusion, formation of large MLV's, lipid intermixing, and leakage of vesicle contents. This effect was reduced significantly when PS or PA was added to the vesicle formulation or when negative charged components were added to the buffer in which the vesicles were prepared. This effect was most pronounced for buffers to which was added the sodium salt of L-(+)-ascorbic acid. Table 1 provides data for vesicles freeze dried in the presence and absence of buffer containing 50 mM ascorbic acid. POPC:TEC-Mal (7:3) vesicles freeze dried in the presence of 50 mM ascorbic acid showed no significant increase in vesicle size following freezing and drying. Leakage of the aqueous marker carboxyfluorescein was also reduced for this vesicle formulation as described below. Addition of pure ascorbic acid alone had no effect on the amount of leakage or vesicle size increase observed for vesicles composed of PC only. Fig. 1 demonstrates the level of carboxyfluorescein retention observed when vesicles of Egg PC:TEC-Mal (7:3) were formed in buffer containing various concentrations of ascorbic acid. At concentrations above 40 mM ascorbic acid, the retention rises to greater than 90% following freeze drying. For all further studies, a concentration of 50 mM ascorbic acid was utilized in all buffers.

FREEZE DRYING (+ AND - 50 mM ASCORBIC ACID)

SAMPLE	+ ASCORBIC ACID	- ASCORBIC ACID
POPC		
BEFORE	49.2+/-13.2 nm	48.8+/-11.8 nm
AFTER	736.6+/-661.2 nm	1430+/-473 nm
POPC:TEC (7:3)		
BEFORE	29.2+/-7.0 nm	27.8+/-6.0 nm
AFTER	2260+/-312 nm	2080+/-387 nm
POPC:TEC-Gal (7:3)		
BEFORE	32.7+/-7.7 nm	33.8+/-7.8 nm
AFTER	954+/-677.2 nm	1930+/-391 nm
POPC:TEC-Mal (7:3)		
BEFORE	55.0+/-23.3 nm	61.3+/-19.3 nm
AFTER	52.4+/-25.9 nm	2140+/-392 nm

Table I: Light scattering before and after.

Fig. 1. Carboxyfluorescein retention in vesicles composed of Egg PC:TEC-Mal (7:3) in the presence of varying amounts of L-(+)-Ascorbic acid. The lipid concentration was maintained at 13 mM.

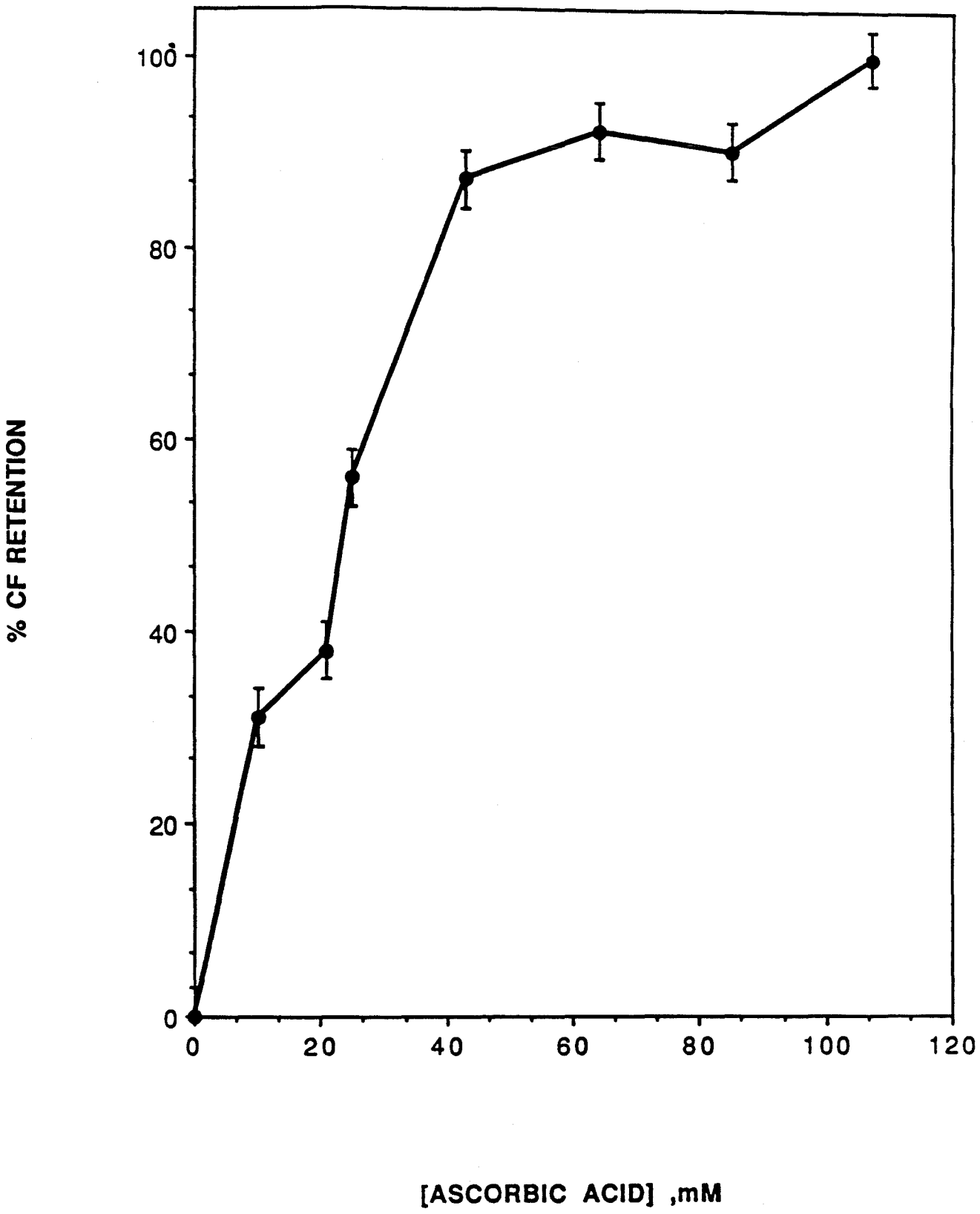

% CF RETENTION
100
80
60
40
20
0
0
20
40
60
80
100
120
[ASCORBIC ACID] ,mM

Fig. 2 demonstrates the amount of carboxyfluorescein retention following freeze drying of Egg PC vesicles with various proportions of the appropriate derivative. For vesicles lacking any additive, virtually complete leakage is observed following freeze drying. When TEC is added to the vesicles in increasing proportions, the level of retention remains below 20%. When TEC-Gal is added to the vesicles, the amount of retention rises and reaches a maximum of 40% at approximately 0.4 moles/mole lipid. The behavior of these compounds is quite distinct when compared to vesicles containing increasing amounts of TEC-Mal. At concentrations between 0.2 and 0.4 moles/mole lipid, the retention of the aqueous marker following freeze drying rises to 100%.

The result obtained with the level of intermixing is paralleled with the behavior observed using the resonance energy transfer assay. Increasing amounts of intermixing as determined from this assay have been correlated with increasing amounts of vesicle fusion. [2] Fig. 3 demonstrates that POPC vesicles containing increasing amounts of TEC-Mal or TEC-Gal result in reductions of the amount of intermixing following freeze drying of the vesicles. This effect is most pronounced with vesicles containing TEC-Mal. Vesicles containing TEC show a slightly enhanced capacity to intermix following dessication and rehydration. As depicted in Fig.4, the results obtained in this assay correlate with the observed retention of carboxyfluorescein following freeze drying.

Fig. 2. Carboxyfluorescein retention for vesicles freeze dried in buffer containing 10 mM TES, 50 mM ascorbic acid, and 0.1 mM EDTA at pH 7.2. The lipid concentration was maintained at 13 mM for all samples. ● TEC, ■TEC-Gal, ▲ TEC-Mal.

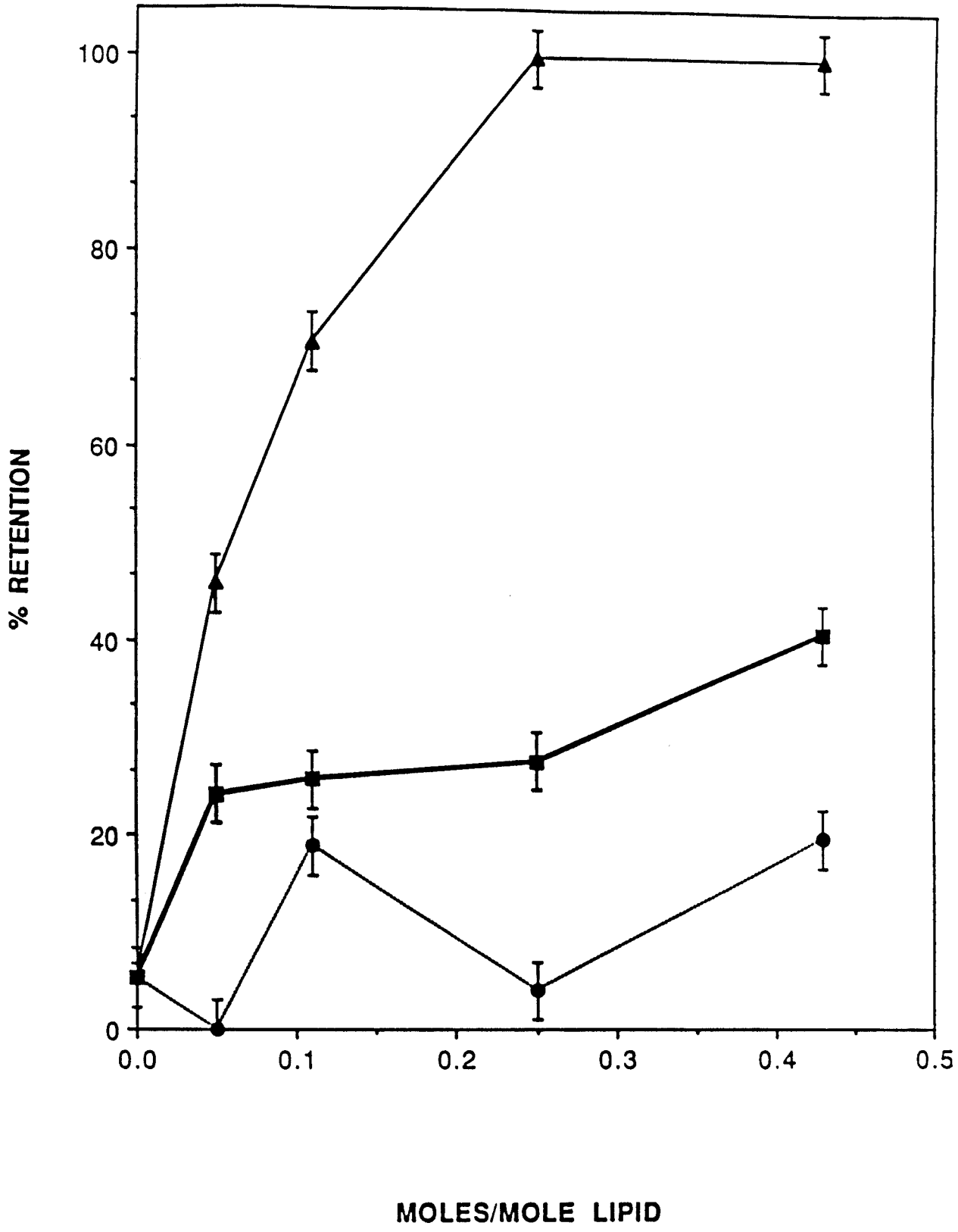

100
80
60
40
20
0
% RETENTION
0.0
0.1
0.2
0.3
0.4
0.5
MOLES/MOLE LIPID

Fig. 3. RET assay for lipid mixing. Vesicles were freeze dried in buffer containing 10 mM TES, 50 mM ascorbic acid, and 0.1 mM EDTA at pH 7.2. Lipid concentration in all samples were maintained at 13 mM. ● TEC ▲ TEC-Gal ■TEC-Mal.

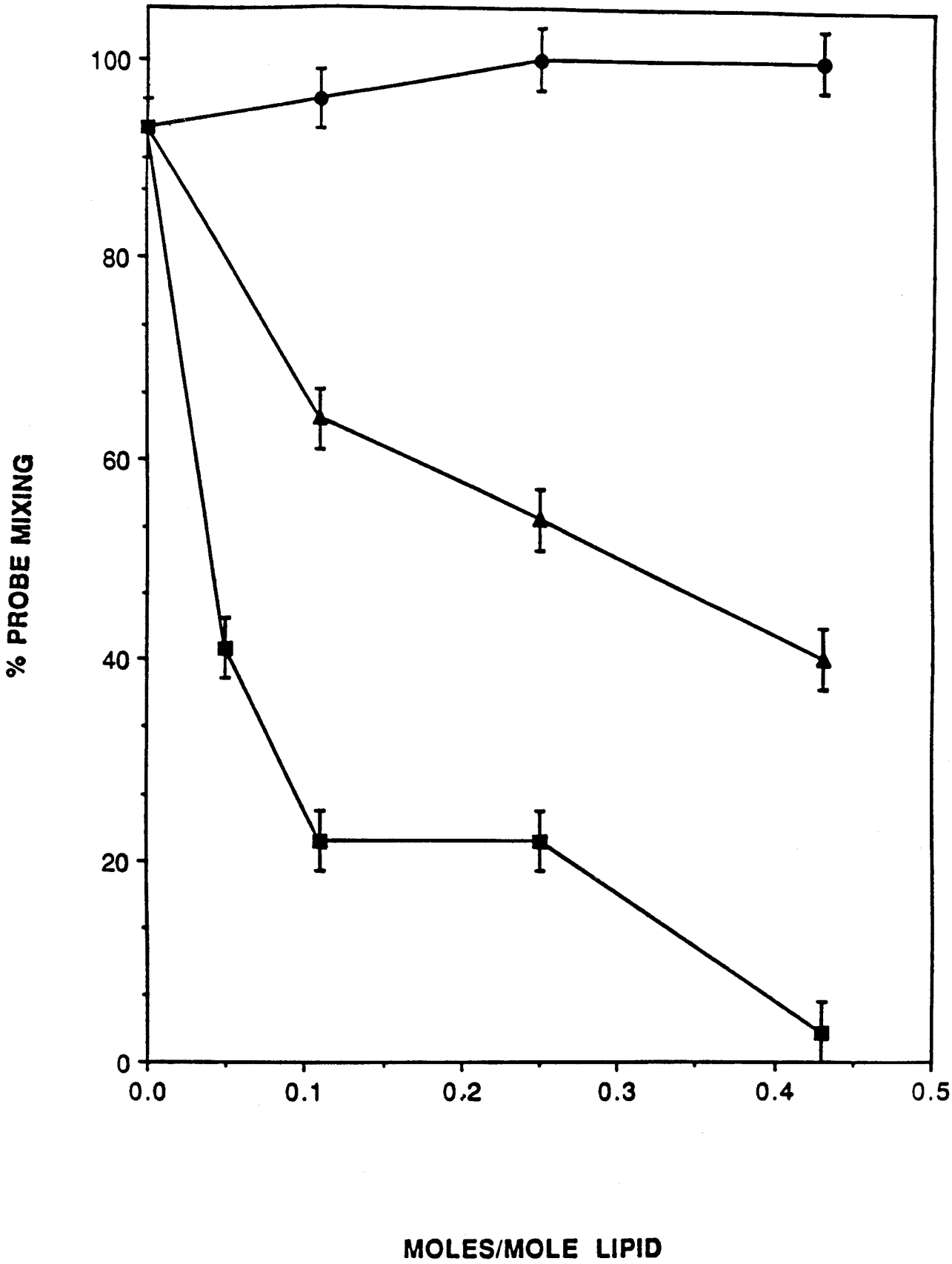
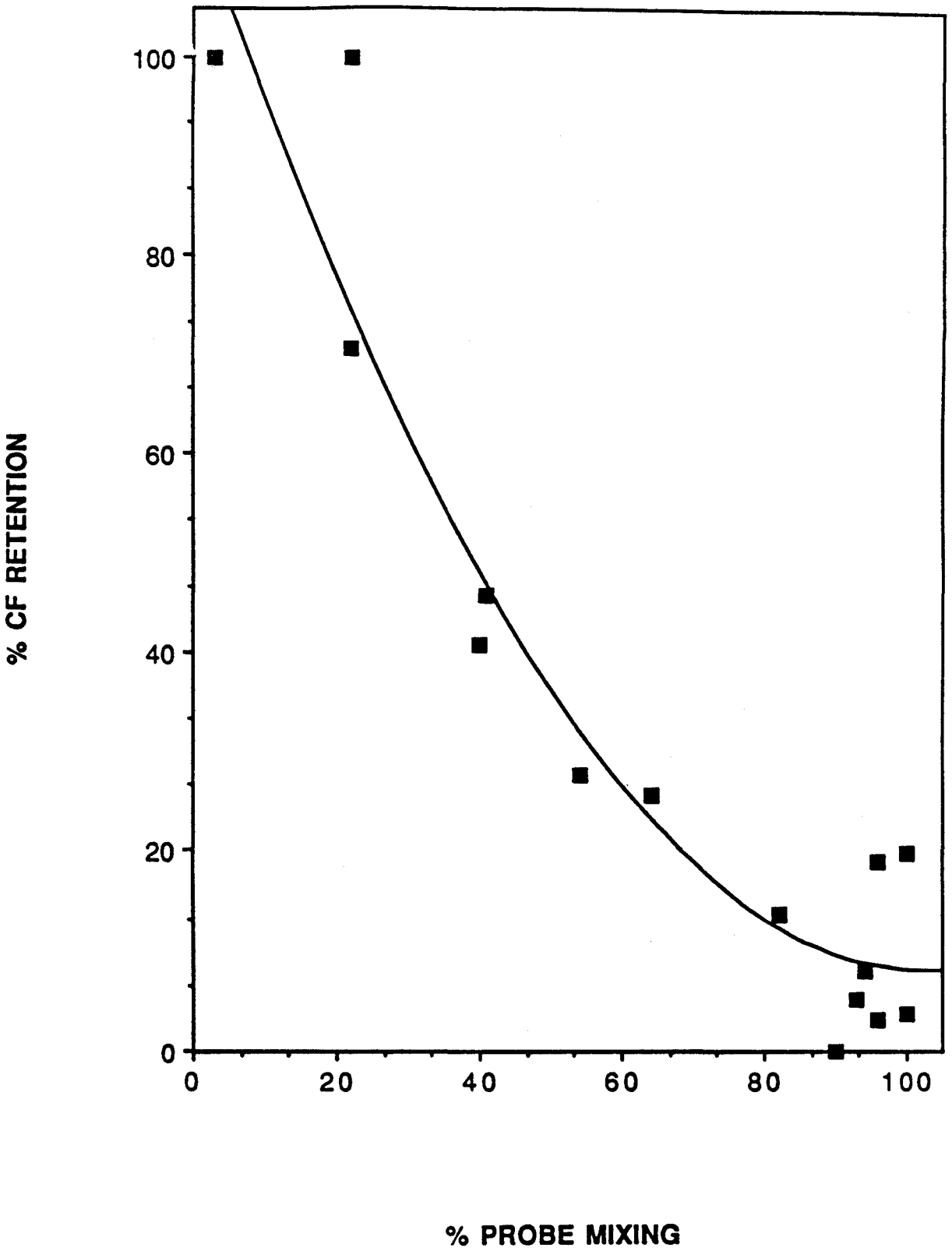

100
80
60
40
20
0
0.0
0.1
0.2
0.3
0.4
0.5
% PROBE MIXING
MOLES/MOLE LIPID

Fig. 4. Correlation of mixing and retention data. Values obtained using the mixing curve were correlated with the corresponding amount of retention observed for the same vesicle preparation. As the results indicate, high levels of probe mixing correspond to low levels of contents retention.


% CF RETENTION
100
80
60
40
20
0
0
20
40
60
80
100
% PROBE MIXING

Because the vesicles produced in the cases described above were formed in the presence of ascorbic acid, it may be assumed that the compound is present on both sides of the bilayer. To test the necessity of having it present inside and outside versus outside only, POPC vesicles with various proportions of TEC-Mal were also made in the absence of ascorbic acid. Following sonication, centrifugation, and column chromatography to remove unentrapped carboxyflourescein, L-(+)-Ascorbic acid was added back to the solution at a final concentration of 50 mM. The results are depicted in Fig. 5. As shown, no discernible difference was observed when the ascorbic acid was added after formation of the vesicles. Because ascorbic acid does not normally penetrate the bilayer, it may be assumed to be located externally only.[12] This result appears to indicate that for the ascorbic acid to be effective, it need only be present in the external medium.

It has been demonstrated previously that the pure derivative TEC-Mal forms vesicles in isolation. The vesicles formed in these studies were prepared with lipid and derivative co-solubilized in chloroform. The capacity of the derivatives to transfer into a pre-formed vesicle was tested in transfer assays. The TLC extract of Egg PC vesicles treated with a suspension of TEC-Mal according to the procedure described under materials and methods, is depicted in Fig. 6, and is indicative of incorporation of TEC-Mal into the vesicles. The R_f values and colors for spots visualized with sulfuric acid (30%) are recorded in Table 2. These vesicles were subsequently subjected to freeze drying. The retention levels

following freeze drying are depicted in Table 3. Retention increased as vesicles were incubated with the TEC-Mal suspension at higher temperatures or for longer time periods at room temperature. This behavior is consistent with a mechanism involving higher levels of retention with greater extents of incorporation of the derivative into the vesicle.

Fig. 5. Comparison of effect of ascorbic acid when present both inside and outside of the vesicles O, as well as on the exterior only ■

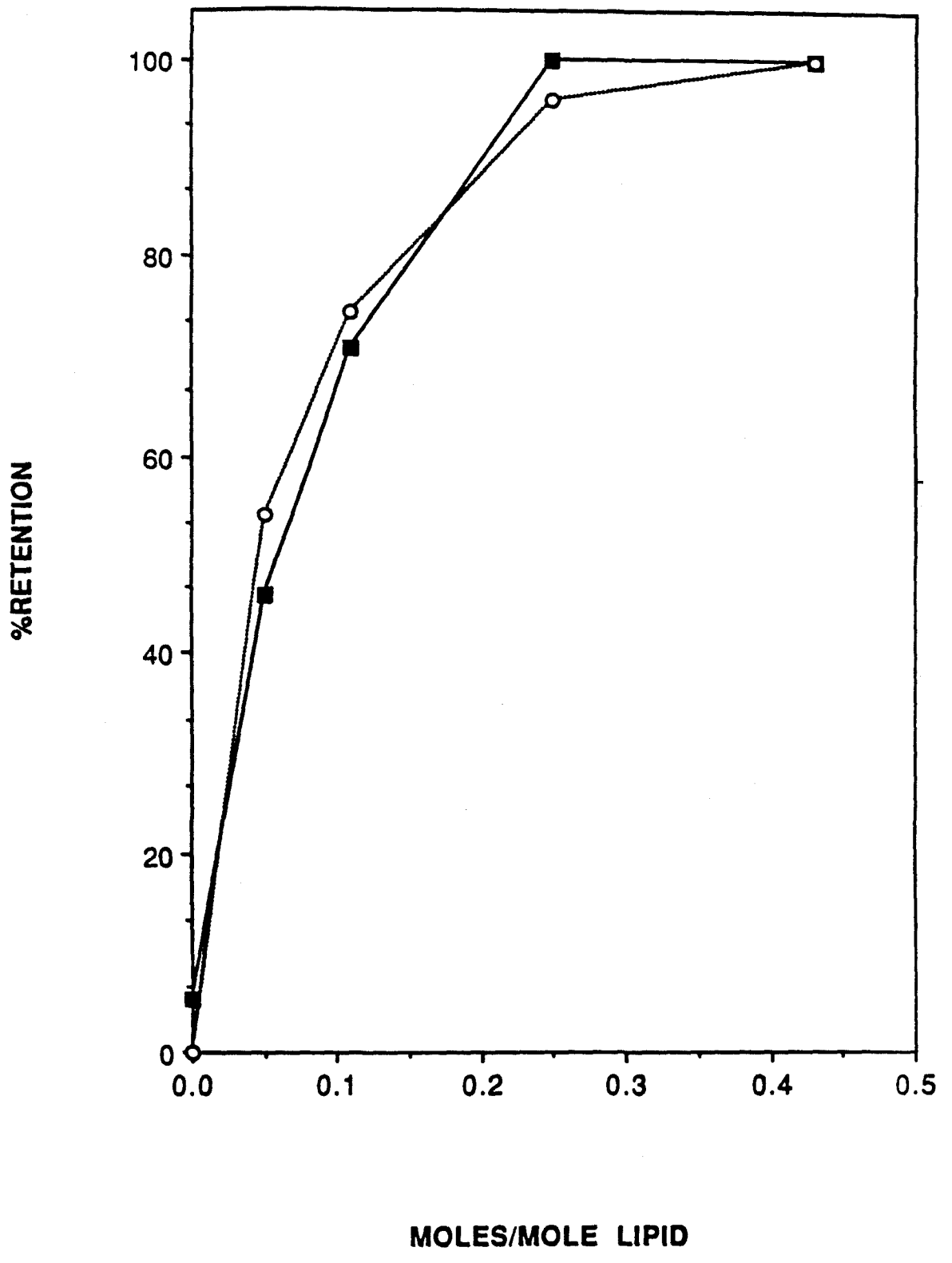
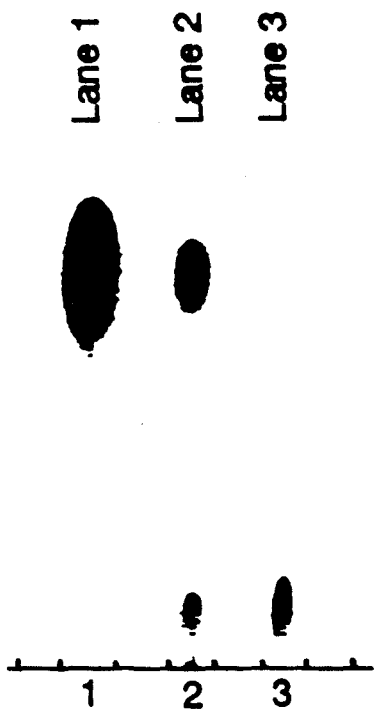

100
80
60
40
20
0
0.0
0.1
0.2
0.3
0.4
0.5
%RETENTION
MOLES/MOLE LIPID

Fig. 6. TLC chromatogram of vesicles extracted with chloroform:methanol 4:1. Samples were visualized using a 30% sulfuric acid solution and heating the plates to develop. The corresponding R_f values and colors of developed spots are recorded in Table II.


Lane 1
Lane 2
Lane 3
1
2
3

VESICLE EXTRACT	R_f	COLOR
A	0.39	pink
B	0.57	brown
EGG PC	0.60	brown
TEC-Mal	0.38	pink

Table II: R_f values and colors.

INCUBATION TEMP.	INCUBATION TIME	% RETENTION
37° C	1 Hour	61.4
47° C	1 Hour	94.2
22° C	3 Hours	80.7

Table III: Transfer assay results.

Conclusions

The results recorded here indicate that a protective capacity is demonstrated for derivatives that tether the carbohydrate to the vesicle bilayer. This protective capacity is dependent on structure. The compound lacking the carbohydrate (TEC) does not protect vesicles against leakage and fusion following freeze drying. Of the two derivatives bearing a carbohydrate moiety (TEC-Gal and TEC-Mal), that bearing the disaccharide (TEC-Mal) demonstrates a more pronounced capacity to protect the membranes against dessication damage. This behavior is consistent with previous observations that monosaccharides demonstrate less of a protective capacity than disaccharides. [10] The differences observed in this case suggest that this diverse behavior must involve specific changes in the extent of interaction of disaccharides versus monosaccharides at the vesicle surface. Such alterations may involve the extent of intercalation of these derivatives into the interfacial region of the lipid and/or the amount of expansion that occurs as a result of this intercalation.

The observation of the necessity of negative charged lipids in vesicle formulations for enhancing the protective action of the carbohydrates is consistent with previous observations by Crowe et al. [7] The results recorded here suggest that this behavior is not only elicited with the addition of negative charged lipids such as PS or PA, but can also be induced through the use of components in the buffer such as ascorbic acid, which also bear a negative charge

at neutral pH. This behavior implies that the phenomena responsible for enhancing the carbohydrate action is determined in part by the environment of the surrounding medium. The presence of negatively charged lipids at the vesicle surface alters the nature of the interface. This alteration can take the form of presenting a more hydrophobic interface.[13] Changes in the environment at the interface would affect the partitioning behavior of the carbohydrate into the interface because such partitioning behavior is determined in part by the entropy and chemical environment of the system. In the same manner, the addition of compounds to the external medium could alter the surrounding matrix in a manner that favors the intercalation of the carbohydrate into the interface. This behavior has been demonstrated for carbohydrates partitioning into polystyrene beads. [14] In this case, alterations in the external environment that disrupted water structure were shown to alter the extent of partitioning into the hydrophobic phase. A second possible explanation for the observed behavior may be one in which alterations in the surface of the vesicles could inhibit intervesicular interactions by providing electrostatic repulsion forces. This alternative mechanism is less feasible because the effect appears to require the presence of particular salts and not simply any negatively charged additive to the solution.

There is a direct negative correlation between the extent of lipid intermixing occuring and the leakage observed for vesicles subjected to freeze drying. During freeze drying, vesicles undergo

fusion events that lead to disruption of bilayer integrity. During this phase, the vesicles are subject to leakage of contents to the external medium. When fusion is reduced to a minimum, the amount of leakage is also reduced to a minimum, suggesting that it is the fusion event that leads to the observed leakage phenomena. The damage sustained by vesicles during freeze drying is thus mediated by fusion and results in the formation of large vesicles lacking their original vesicle contents. The carbohydrate action ameliorates this effect by reducing the amount of vesicle fusion. In the presence of the carbohydrates, the lipid mixing is negligible, leakage does not occur, and vesicle size is maintained. With the derivatives studied here, the stoichiometry of Carbohydrate:Lipid required to provide protection is on the order of 0.5 mole carbohydrate/mole lipid. For vesicles that are not tethered to the vesicle, similar behavior is observed for Carbohydrate:Lipid ratios on the order of 2.0 mole/mole lipid. [10] This indicates that for the tethered compounds, there is an approximate reduction in the stoichiometry by a factor of 4. This result appears to indicate that it is the carbohydrate at the interface, which is responsible for producing the protective action, and not the carbohydrate in the bulk phase. Tethering of the carbohydrate to the interfacial region reduces the amount of carbohydrate required to a minimum. In this instance, an excess bulk phase is not present. This is borne out further by considering that, if one assumes an average vesicle radius of 250 Å, then the surface area of a vesicle would be on the order of 7.8×10^5 Å^2. For an average phospholipid, the surface

area occupied is approximately 49 $Å^2$.[15] This would therefore require 16,000 molecules of lipid to comprise the surface area of a single vesicle's outer leaflet. Assuming a surface area of 30 $Å^2$ per carbohydrate (disaccharide), this would require a total of 26,000 molecules to cover the surface.[16] This is a molar ratio of 1.6:1 (Carbohydrate:Lipid), a value in rough agreement with the stoichiometry reported to afford protection to liposomes dried in the presence of trehalose.

Finally, the transfer capacity of the derivatives from the bulk phase into the lipid phase is demonstrated by a capacity to incorporate into pre-formed liposomes. With the observed transfer, there is also the induction of the capacity to retain contents following freeze drying. This implies that the amount of derivative incorporating into the vesicles is on the order of at least 0.5 mole/mole lipid where retention is highest. The transfer of the agents from the bulk phase where they exist in the form of pure derivative bilayers, to the lipid vesicle, is governed by time and temperature, indicating a direct kinetic behavior with respect to the rate of transfer. Whether this rate is first or second order has not yet been determined.

References

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16. This value was obtained by constructing ball and stick models and measuring the corresponding length and width of the molecule. In this manner, the carbohydrate is treated as a rectangular object with a length of approximately 10Å and a width of approximately 3 Å.

CHAPTER 4

Phase Behavior of Novel Synthetic Glycolipids in Aqueous Solution

The results recorded in Chapters Two and Three indicate pronounced cryo and lyoprotective capacity for the compounds under study. As outlined previously in the introduction, however, the efficacy of the compounds described in Chapters 1-3 must depend directly on their incorporation into a bilayer structure without disruption of the membrane integrity. This ability is in turn governed in part by the phase properties of dispersions of the pure derivative.

The shape of a molecule and its propensity to form lamellar or micellar phases dictates the alterations observed when added to a membrane formulation.[1,2] For instance, compounds such as naturally occuring gangliosides that form micellar phases tend to disrupt bilayer integrity when added to membranes in high concentrations.[3,4,5] This behavior has been correlated with a hypothesis in which the shape of a molecule dictates its capacity to form lamellar, micellar, or cubic phases in isolation as well as to induce these phases when combined with phospholipids. [5] Therefore, in order to understand the full extent of the interactions of the carbohydrate derivatives with liposomal membranes and determine their overall efficacy in this regard, it was necessary to examine the range of their phase behavior in isolation.

Electron micrographs of aqueous suspensions of the derivatives are characteristic of bilayer assembly. Fig. 1 depicts hand shaken suspensions of TEC-Gal in NTE buffer at pH 7.4. [6] Samples were stained for electron microscopy using 1% uranyl acetate. The

lamellar repeat distance estimated from these photos is on the order of 62.8+/-2.2 Angstroms.[7] The bilayer thickness can also be estimated at approximately 37 Angstroms. Brief sonication (<10 min.) of the suspensions in a bath sonicator results in the formation of oligolamellar vesicles (Fig. 2) of approximately 5 microns in diameter. Further sonication of the TEC-Mal derivative results in the formation of a clear solution consisting of unilamellar vesicles. The size of these vesicles was determined, using laser light scattering, to be 56.0+/-6.1 nm.[8] Unilamellar vesicles were not observed for samples of TEC-Gal even after extensive bath sonication. In this case, there was only a noted propensity to form aggregated material.

The ability of the vesicles formed to entrap an aqueous marker was also examined. This serves as a test of the compound's ability to form closed vesicular structures. The method described by Weinstein et al. [9] was utilized in this study to demonstrate the capacity to entrap the fluorescent compound carboxyfluorescein. This compound normally self-quenches at high concentrations such as 100 mM but fluoresces intensely when the dye is diluted.

Fig. 1. Electron micrographs of dispersions of TEC-Gal in NTE buffer at pH 7.4. (Top) Hand shaken dispersion of vesicles stained with uranyl acetate (1% w/v). (Bottom) Sonicated dipersion of TEC-Gal in NTE buffer at pH 7.4 stained with uranyl acetate. The suspension was sonicated for 10 minutes in a bath sonicator. This results in the formation of oligolamellar vesicles on the order of 5 μm in size. Mag 240,000X. Bar is approximately 0.4 microns.

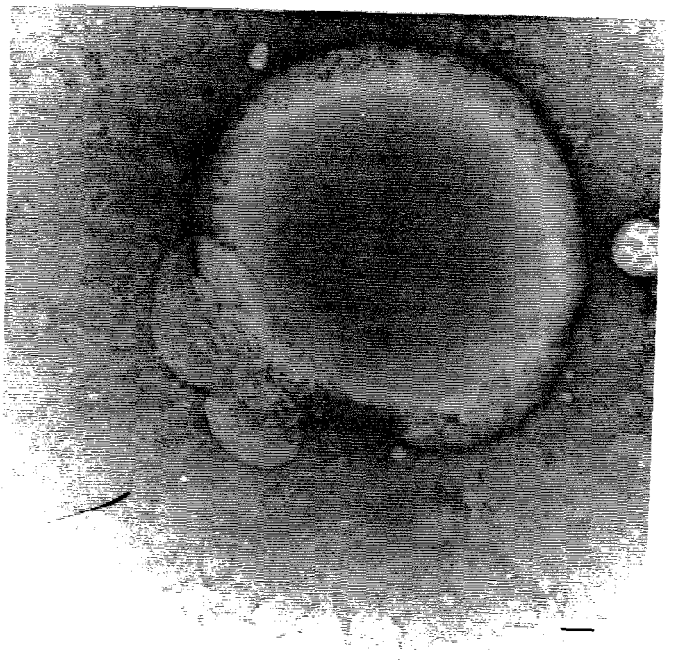
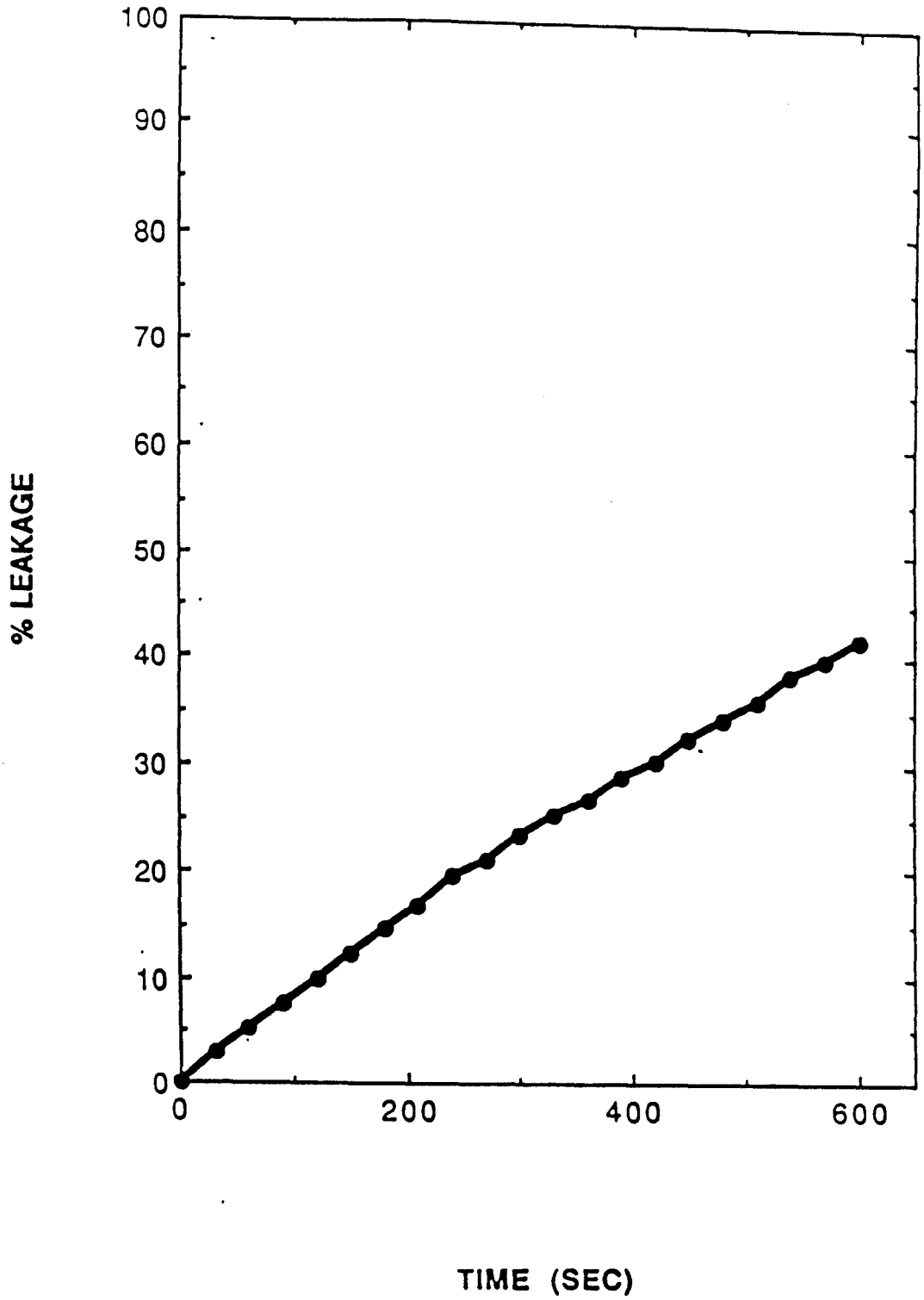


Fig. 2. Carboxyfluorescein release from TEC-Mal SUV's as a function of time. Release was monitored as a function of time. Vesicles were dissolved with rat or calf serum (5% final concentration) in order to obtain the 100% lysis value.

CARBOXYFLUORESCEIN LEAKAGE FROM TEC-Mal SUV'S



Samples of TEC-Gal and TEC-Mal were sonicated in HEPES buffer at pH 7.4 to which was added 100 mM carboxyflourescein. Unentrapped marker was removed by passing the vesicles over columns of Sephadex G-25-50 using the HEPES buffer as eluant . Carboxyfluorescein leakage was monitored as a function of time.[10] The 100% value was determined by lysing the vesicles with serum.[11] There was no indication of entrapment of the dye for vesicles composed of TEC-Gal. As demonstrated in Fig.2, this was not the case for the TEC-Mal derivative. In this instance, there is a definite entrapment of the carboxyfluorescein that subsequently leaks from the vesicles with time. The rate of leakage is rapid (>40% in 5 minutes), indicating that the membranes formed, while providing some barrier to leakage, are not completely impermeable.

The observations recorded here are consistent with a lamellar phase arrangement for the TEC-Gal and TEC-Mal compounds in isolation. This behavior is consistent with the prediction made for the phase behavior using the shape hypothesis and assigning both compounds a cylindrical shape. Such compounds normally form bilayer structure in aqueous solution. In this case, packing considerations appear to make the formed vesicles leaky to entrapped aqueous markers. It is interesting to note that the behavior of the mono- and disaccharide derivatives differ in the extent to which this leakiness occurs. This behavior is similar to that observed for mono- and disaccharide derivatives of diacyl glycerols and could reflect on differences in hydration behavior between the two compounds. [12]

This behavior explains in part the observations made in Chapter One, in that addition of the derivatives to aqueous suspensions of DOPE, DOPC, and DPPC stabilizes bilayer structure while reducing bilayer packing (i.e., fluidizes the membrane). It is this feature of lamellar phase formation in isolation, which makes possible the large-scale addition of these compounds to lipid vesicles noted in Chapters 1-3, where maintenance of bilayer structure is essential.

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6. Vesicles were prepared for EM as follows: Samples were dried from chloroform stock solutions under nitrogen and subsequently under mechanical pump vacuum for 24 hours to provide approximately 2 mg of the derivative in the dry form. The samples were then hydrated with 1 ml NTE buffer (100mM NaCl, 10 mM Tris-HCl, 0.2 mM EDTA, pH 7.4) and vortexed for 30 seconds. A sample of 10 µl of this suspension was then applied dropwise to a carbon coated copper grid. This was followed by a drop of a 1% (w/v) solution of uranyl acetate used as a negative stain. Micrographs were obtained using a Philips EM 201 electron microscope operating at 80 Kv. Sample magnifications are recorded in the appropriate figures.

7. This value is in rough agreement with that predicted for a fully extended molecule. The estimated length of the sterol portion

is approximately 15 Angstroms. The estimated length of the hydrophilic linker and carbohydrate group is approximately 17 Angstroms. This leads to a predicted bilayer thickness of 30 Angstroms and a lamellar repeat distance of 64 Angstroms.

8. Light scattering was performed using a Malvern 4700c sub micron particle analyzer. Excitation was carried out with a Spectra physics argon ion laser at 488 nm. The intensity of light scattered was monitored via a detector oriented at 90 degrees relative to the excitation beam. Sample concentration was at 3.8 mM in HEPES buffer (0.01M Hepes, 120 mM NaCl, pH 7.4).

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10. Fluorescence intensity changes were monitored using the SLM 4800 spectrofluorometer with excitation at 490 nm and emission at 520 nm.

11. Vesicles were dissolved in the presence of rat or calf serum (5% final concentration) to yield the total amount of entrapped carboxyfluorescein.

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CHAPTER 5

Alterations in Membrane Surfaces Induced by Attachment of Carbohydrates

Abstract

We have examined the behavior of the dry phospholipid dipalmitoyl phosphatidylcholine (DPPC) in the presence of several carbohydrate derivatives. These carbohydrate derivatives possess a hydrophobic portion that is incorporated directly into the DPPC membrane, and a hydrophilic portion that places the carbohydrate structure at the membrane interface with the surrounding matrix. In the presence of these derivatives, the physical properties of the membrane are altered. These alterations are evident in changes observed in the phosphate mode of the phospholipid portion of the membrane. In addition, the phase transition behavior of the phospholipids in the acyl chain region is significantly altered as evidenced by the reduction in the gel to liquid crystalline phase transition temperature. These results are consistent with those previously reported for free carbohydrates interacting with membranes in which a water replacement hypothesis has been used to explain the behavior. The attachment of carbohydrates to the membrane enhances this effect and localizes the specific alterations at the membrane interface.

Introduction

In Chapters 1-4, the interaction of the derivatives under study with liposomal membranes has been documented. Most significantly, their ability to protect membranes against dehydration-induced damage has been described. The question to be resolved, however, is the nature by which this protection is afforded.

Several theories have developed to explain the mechanistic details by which carbohydrates interact and protect membranes against damage. Foremost among these, as outlined previously in Chapter Two, is the water replacement hypothesis forwarded by Crowe. Most recently, Lee et al. [1,2] have demonstrated that the addition of trehalose to DPPC results in a lowering of the phase transition temperature and formation of two new lipid phases. These are represented by the L_κ and L_λ phase and correspond in character to the L_β and L_α phases for lipids in the absence of carbohydrate. X-ray diffraction of the L_κ phase shows evidence of an expanded gel phase bilayer structure with excess crystalline trehalose. This phase occurs below 54° C for a trehalose:lipid mole ratio of 2:1. At temperatures above 56° C, the acyl chains become disordered along the length of the chain, characteristic of the L_α phase.

Lee et al. have postulated that it is the intercalation of the carbohydrate into the interfacial region, that induces the

alterations noted above via expansion of the lattice and modification of the whole lipid interfacial region. This theory deviates from the classical H_2O replacement hypothesis by suggesting that it is intercalation per se and not necessarily direct bonding that is responsible for the observed effects. If this hypothesis is correct, then direct incorporation of the carbohydrate in the case of the derivatives into the interfacial region should induce the same effects. In this section, the direct interactions of the derivatives with vesicles of DPPC are examined.

Materials and Methods

Lipids were purchased from Avanti Polar Lipids and used without further purification. The compounds depicted in Fig.1 of the introduction were prepared according to methods described earlier, but with several modifications .

Synthesis of Carbohydrate Derivatives

The TEC derivative was prepared according to methods described previously.[3] For synthesis of TEC-Mal, a procedure was utilized in which TEC, mercury bromide, mercury oxide, and calcium sulfate in chloroform was added to a round bottom flask. The mixture was stirred for 30 minutes. Subsequently, the Acetobromo derivative of the appropriate sugar was added to the solution with stirring. The reaction mixture was then stirred in the dark for 3-4 days. The mole ratio of Sterol/mercury oxide/mercury bromide/sugar was maintained at 1:1:0.04:1. At the

end of the 3-4 day period, the mixture was filtered and solvent evaporated to yield a white solid. This material was taken up in chloroform:ethyl acetate 1:1 and eluted on a silica gel column. Fractions were assayed via TLC. Several fractions were combined and solvent removed by evaporation to yield a white solid. This material was dissolved in methanol to which was added sodium methoxide (0.05 M). The solution was stirred for several hours at room temperature. The solution was then filtered and solvent evaporated to yield a white solid. The compounds were examined via IR, NMR, mp,mass spec, and TLC. The use of the mercury salts in place of the silver salts used previously resulted in a 3-4 fold increase in the reaction yield .

Differential Scanning Calorimetry

DSC was performed on the Hart Microcal calorimeter or Perkin-Elmer DSC 7. Samples were dissolved in chloroform and dried for 24-48 hours under vacuum. All samples were maintained under dry nitrogen throughout the process. Sample pan loading was carried out in a glove box flushed with dry nitrogen. Scans were recorded at a rate of 30°C per hour on the Microcal or 20°C per minute on the DSC 7. Pure DPPC SUV's prepared in this manner have exhibited transitions as high as 102°C, well above the 64°C transition normally associated with the dihydrate.[4,5] Samples that possess less than the water normally associated with the dihydrate but that are not completely anhydrous, normally display phase transitions between 64° and 102°C. All of the samples used in

these studies were assayed via DSC and consistently yielded phase transition temperatures in the range of 77°C for the pure lipid, a value indicative of the "dihydrate" of DPPC.[4,6,7]

Fourier Transform Infrared Spectroscopy

Samples for FT-IR were run on the Perkin-Elmer model IR-681 spectrometer or Mattson model FT-IR. Each sample was prepared by drying from chloroform under vacuum for 24-48 hours. The material to be examined was placed on barium fluoride windows in a glove box flushed with dry nitrogen. Heating and cooling was carried out using a Peltier device. Temperature changes were monitored via a thermocouple attached to the sample cell holder. Approximately 50-75 transients were recorded per sample. Spectra were baseline corrected and smoothed using a 13 point smooth formula. Spectra in the 3000-3200 cm^{-1} region were used to monitor levels of residual moisture. The intensity of peaks in this region for materials containing carbohydrate, when corrected for the presence of carbohydrate OH groups,was no different than that obtained for pure lipid.

Raman Spectroscopy

Raman spectra were collected on a Spex Ramalog spectrometer. A Spectraphysics argon-ion laser operating at 488 nm was used as the excitation source. Spectra were smoothed using a 3 point method. For sample preparation, lipid was dried from chloroform under vacuum for 24-48 hours. Dry material was then transferred in a glove box flushed with dry nitrogen to capillary tubes of 1 mm diameter. The capillary tubes were subsequently sealed under flame. All Raman samples prepared in this manner were assessed for residual moisture by thermal gravimetric analysis using a Perkin-Elmer TGA work station. Changes in weight were recorded as the sample was heated from room temperature to 120°C at a rate of 20 degrees per minute. The dihydrate of DPPC showed a 2-4% weight loss when treated in this manner and corresponded to a sample exhibiting a phase transition at 77°C as determined by DSC. No detectable change was observed for samples used in the Raman study. All spectra were recorded at room temperature with a step increment of $0.75cm^{-1}$ and a resolution of 1 cm^{-1}. For samples in the carbonyl region, 30-40 transients were recorded due to the low intensity of this band in Raman.

RESULTS AND DISCUSSION

IR and DSC

Fig. 1 depicts spectra obtained in the phosphate asymmetric stretching region for samples of DPPC with various additives. Pure DPPC exhibits a phosphate asymmetric peak at 1252 cm^{-1}. This peak remains at 1252 cm^{-1} with addition of cholesterol or TEC (7:3 mole ratio of Lipid:Derivative) . With addition of TEC-Mal, this peak is shifted to 1239 cm^{-1}. With heating of this sample (Fig.2), a shoulder grows into a distinct peak at 1252 cm^{-1}. A plot of the ratio of the intensity of this peak to that at 1239 cm^{-1} shows a break at approximately 42° Celsius (Fig. 3). Cooling of the sample leads to the disappearance of the peak at 1252 cm^{-1}. These peak positions are summarized in Table One.

IR was also used to follow the gel to liquid crystalline transition of the phospholipid acyl chains. Results are summarized in Fig.4. A plot of the position of the methylene asymmetric stretch position versus temperature indicates a break at approximately 50° C for DPPC:TEC-Mal (7:3). As indicated in Table Two, this result is confirmed in DSC. The observed transition is well below the transition at 65° Celsius normally associated with the DPPC dihydrate.[4] Spectra of each of the pure derivatives alone (TEC-Mal and TEC) show no interfering bands in the region of interest over this temperature range. The recorded transition is in agreement with that observed by Lee et al. for TRE:DPPC in a mole ratio of 2:1 (i.e., 49 +/-2° Celsius), as well as with the phase

transition temperature observed for samples of TRE:DPPC (2:1) dried from aqueous solutions (47-53°C).[6,8] These results would indicate that at temperatures below 51° Celsius, the lipid exists in the gel-like phase designated as L_k by Lee et al. The induction of the L_K phase is brought about in this case , however, by the addtion of as little as 0.42 moles of the carbohydrate/mole lipid. This represents a decrease by a factor of 4.7 in the stoichiometry.

The shifts in the phosphate region also correspond to those normally associated with the addition of water to dry DPPC.[9] In this case, there is also a unique temperature dependence on the position of the phosphate band for samples containing the cabohydrate derivatives. The shift to lower wavenumbers of this phosphate asymmetric stretching mode upon addition of water is normally assoicated with the hydrogen bonding of water to the phosphate group or reorientation of this group.[10,11] In this instance, only derivatives bearing a carbohydrate moiety are capable of inducing this effect. This behavior is consistent with a mechanism involving hydrogen bonding of the carbohydrate -OH groups to the phosphate group of the phospholipid. Increasing temperature should lead to breaking of the hydrogen bonds. The unbonded phosphate should then be equivalent to a phosphate in which no carbohydrate or water is present. Subsequent cooling should lead to re-formation of these bonds as long as phase separation has not occurred. This would explain the reversible nature of the shift observed in the phosphate mode with temperature and could also explain the restricted motion normally

observed for the phosphate segment in the presence of carbohydrates.[1]

Fig. 1. Infrared spectra in region of the phosphate asymmetric stretching mode. Lipid: Derivative mole ratios in each case were 7:3. Spectra were recorded at room temperature.

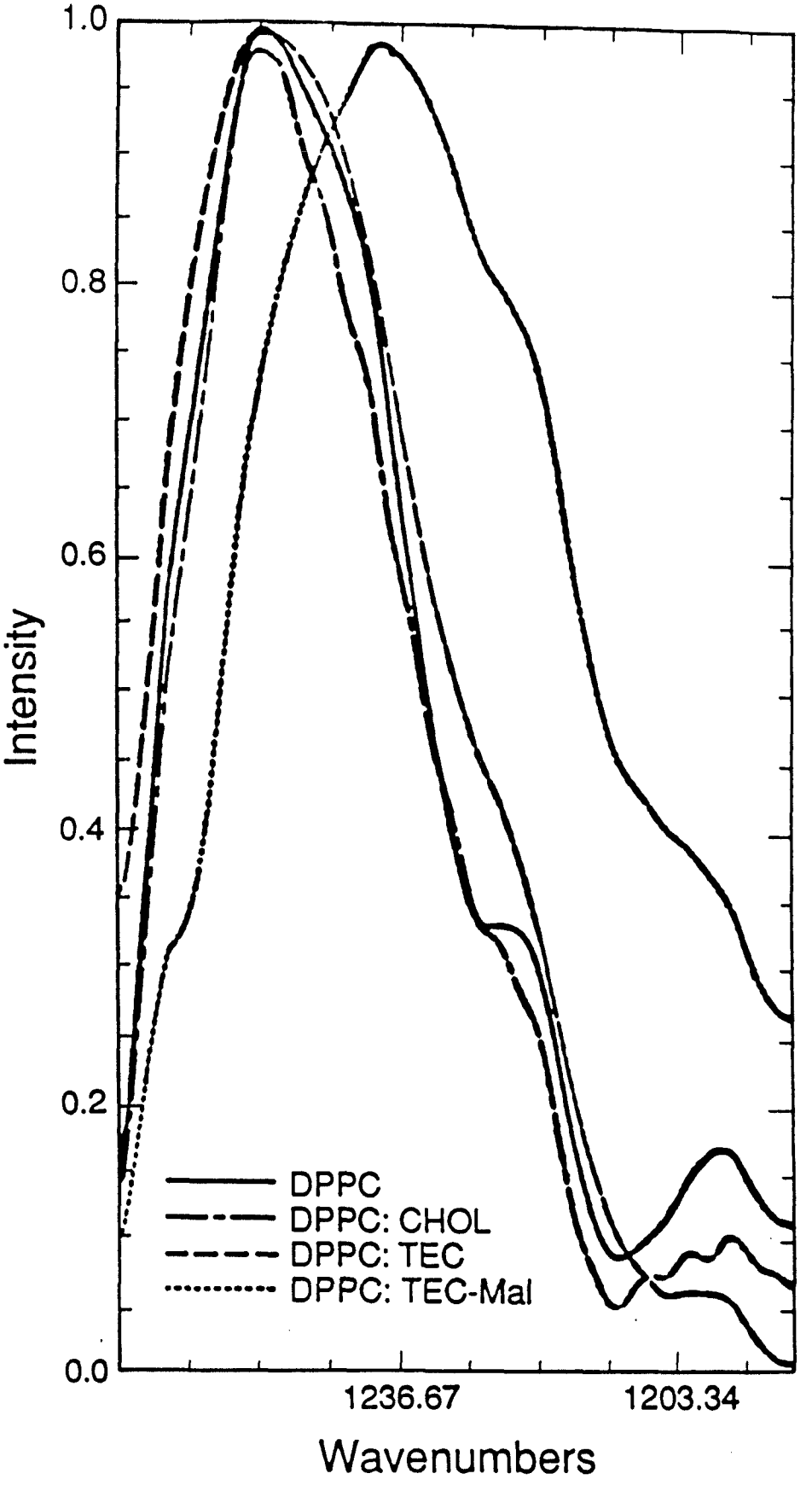

1.0
0.8
0.6
0.4
0.2
0.0
Intensity
DPPC
DPPC: CHOL
DPPC: TEC
DPPC: TEC-Mal
1236.67
1203.34
Wavenumbers

Fig. 2. Spectra in the phosphate asymmetric stretching region as a function of temperature for DPPC:TEC-Mal (7:3). Spectra were recorded over the temperature range of 22-65^{o}C in increments of 2-5 degrees.

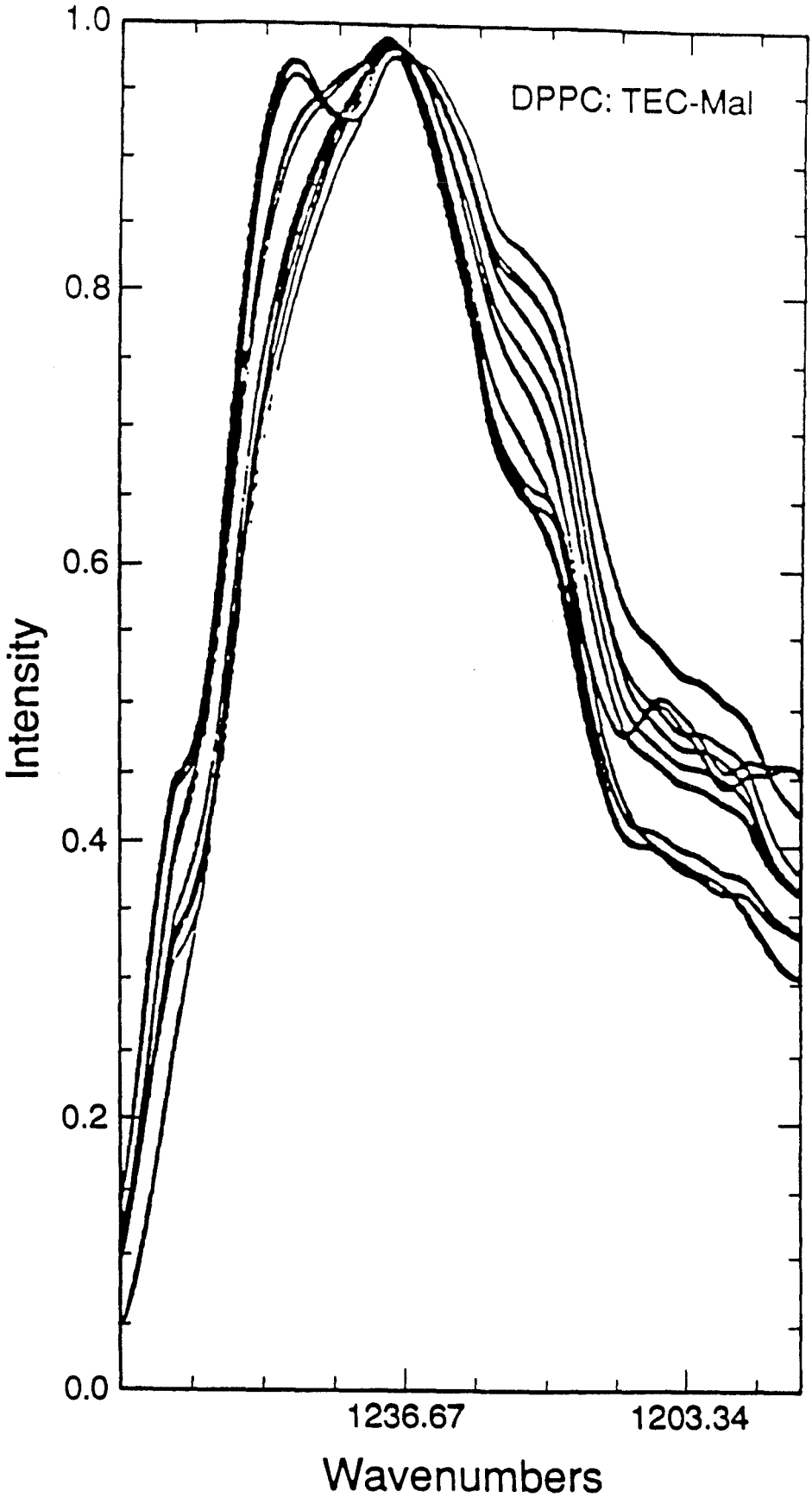
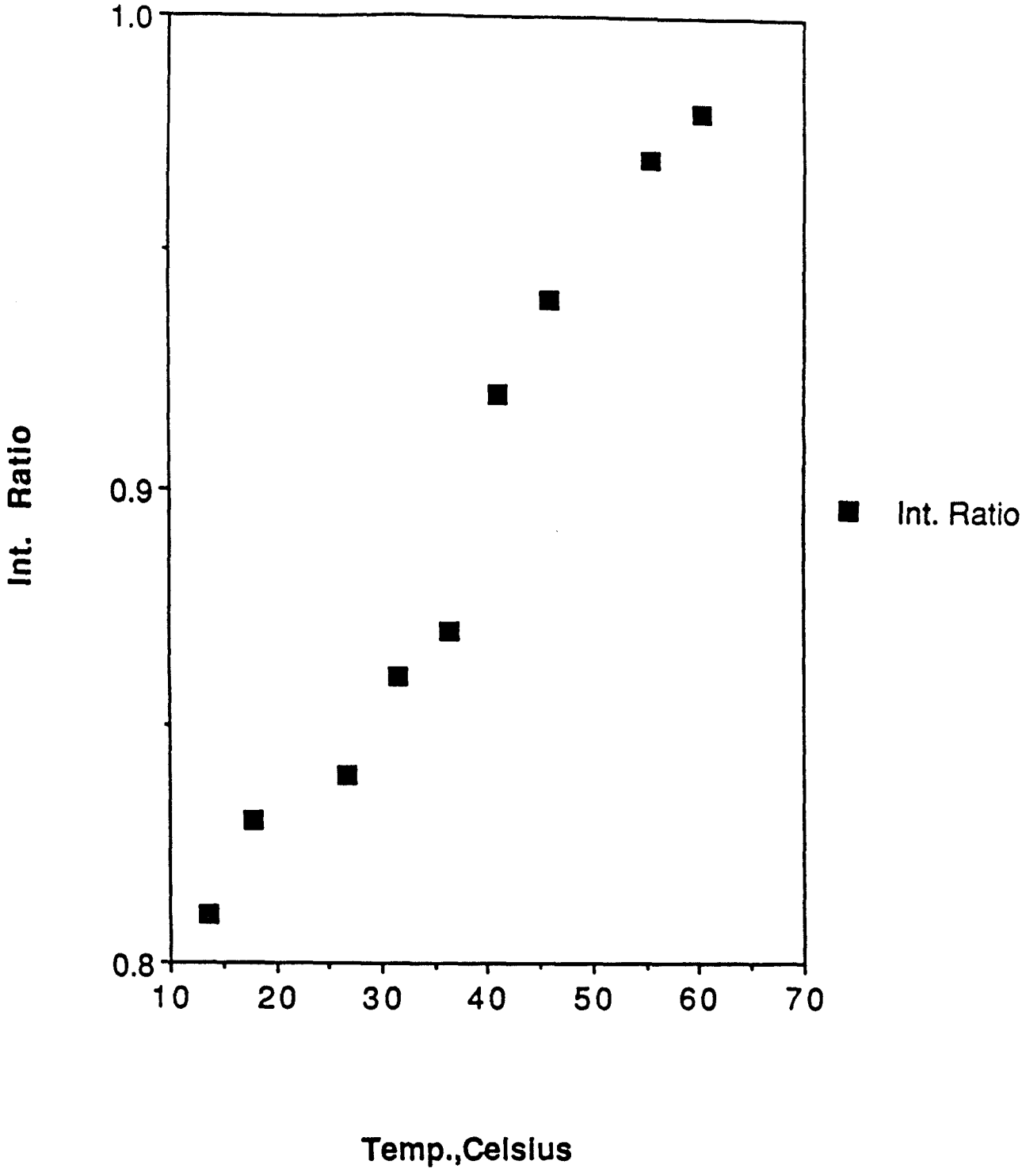

DPPC: TEC-Mal
1.0
0.8
0.6
0.4
0.2
0.0
Intensity
1236.67
1203.34
Wavenumbers

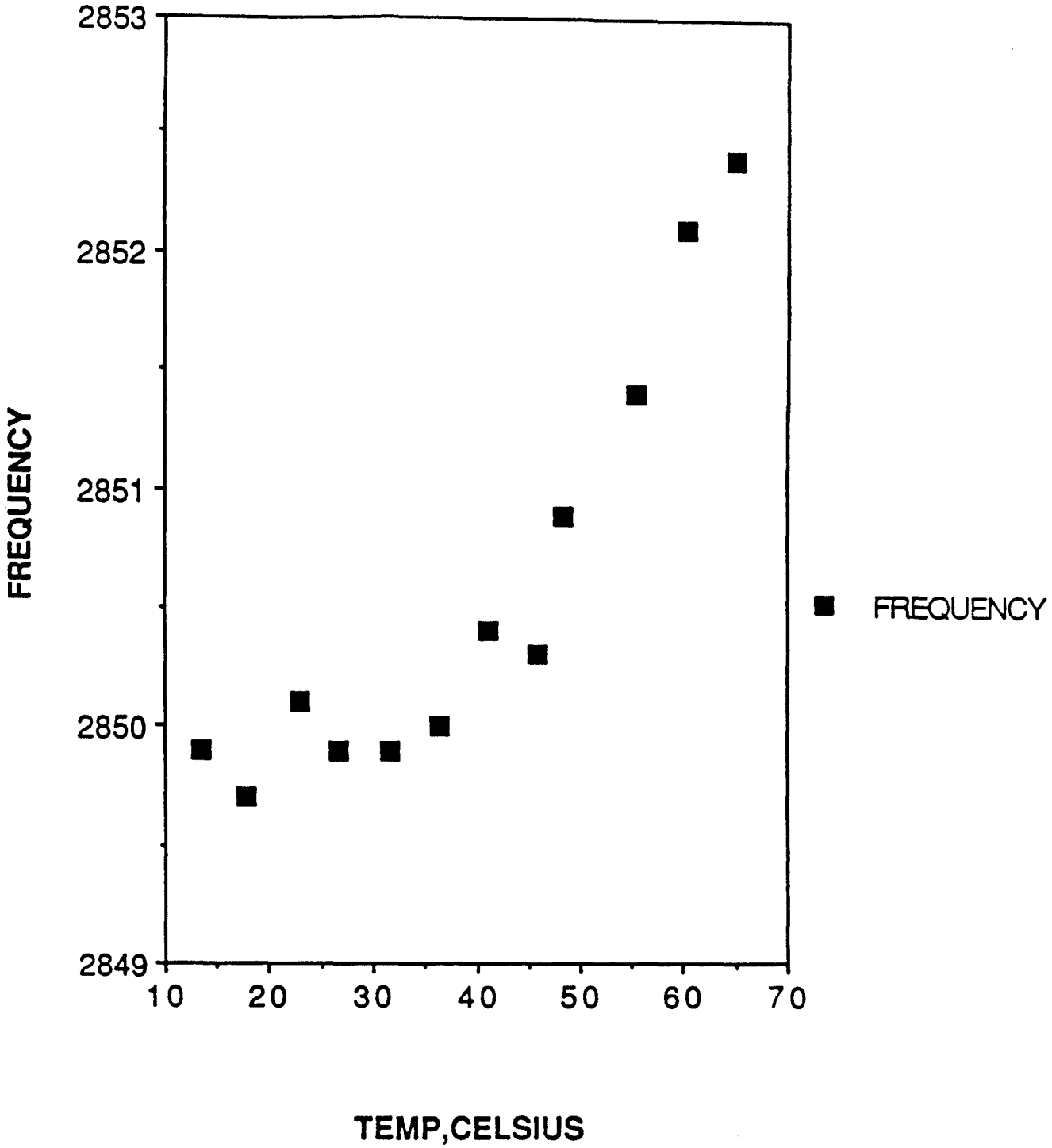
Fig. 3. Intensity ratio of 1252/1239 cm^{-1} bands as a function of temperature for DPPC:TEC-Mal (7:3).


1.0
0.9
0.8
Int. Ratio
10
20
30
40
50
60
70
Temp.,Celsius
Int. Ratio

	Temperature	
Sample	22°C	60°C
DPPC	1254	1254
DPPC:CHOL	1254	1254
DPPC:TEC	1254	1254
DPPC:TEC-Mal	1240	1240,1254

Table I: Phosphate peak positions.

Fig. 4. Position of methylene asymmetric stretching mode as a function of temperature for DPPC:TEC-Mal (7:3).


2853
2852
2851
2850
2849
FREQUENCY
10
20
30
40
50
60
70
TEMP,CELSIUS
FREQUENCY

Sample	DSC	FT-IR
DPPC	77°C	- -
DPPC:TEC	77°C	- -
DPPC:TEC-Mal	51°C	50°C

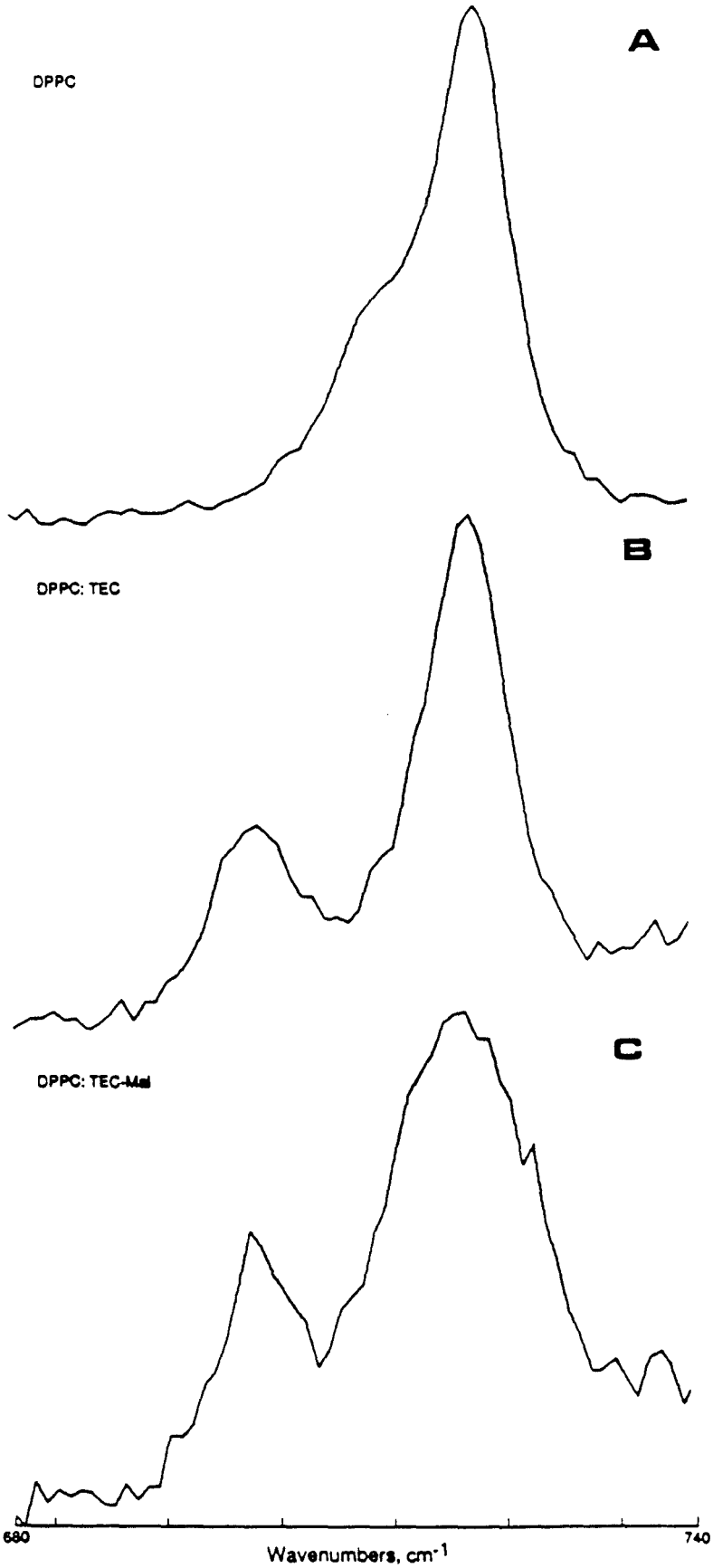
Table II: Phase transition temperatures.

Raman Spectroscopy

Raman spectroscopy was used to probe the lipid interfacial and acyl chain region. Spectra in the 680-740 cm^{-1} region are depicted in Fig. 5. Dry DPPC possesses a single peak at 721 cm^{-1} and a distinct shoulder at 713 cm^{-1}. The intensity and position of these bands are known to correspond to the hydration level and phase state of the bilayer. [12] In particular, the intensity of this band remains relatively constant over a wide range of temperatures. For this reason, it is often used as an internal standard. It has been observed, however, that there is a reduction in intensity of this band by about 15% as the system goes from the pre-transition to the L_α phase. This effect has been correlated with an expansion and reorganization in the interfacial region, which occurs at the pre-transition. [13]

For DPPC:TEC (7:3) and DPPC:TEC-Mal (7:3), the band positions observed for pure DPPC remain unchanged in the 680-740 cm^{-1} range. There is, however, an additional band at 702 cm^{-1} assigned to a vibrational mode of the sterol backbone.[12] This peak thus serves as an internal standard for comparing intensities in this region. As depicted in Table Three, when the ratios of the 721/702 cm^{-1} peaks are compared, there is a distinct reduction by 15% in the intensity of the 721 cm^{-1} peak when TEC-Mal is present relative to CHOL. The TEC derivative actually shows an increase in this ratio when compared to CHOL. The alteration observed for

Fig. 5. Spectra in the 680-740 cm^{-1} region corresponding to the C-N asymmetric stretching mode. (A) DPPC, (B) DPPC:TEC (7:3), (C) DPPC:TEC-Mal (7:3). Spectra were recorded at room temperature.


A
DPPC
B
DPPC: TEC
C
DPPC: TEC-Mal
680
740
Wavenumbers, cm-1

Additive	Peak Ratio 721/702
TEC	2.62
TEC-Mal	1.73
CHOL	2.00

Table III: Intensity ratios in C-N region.

TEC-Mal is consistent with a state of the membrane in which the interface is expanded. This is comparable to the state that exists after passage through the pre-transition, an observation that is also confirmed by examination of interchain disorder as discussed below.

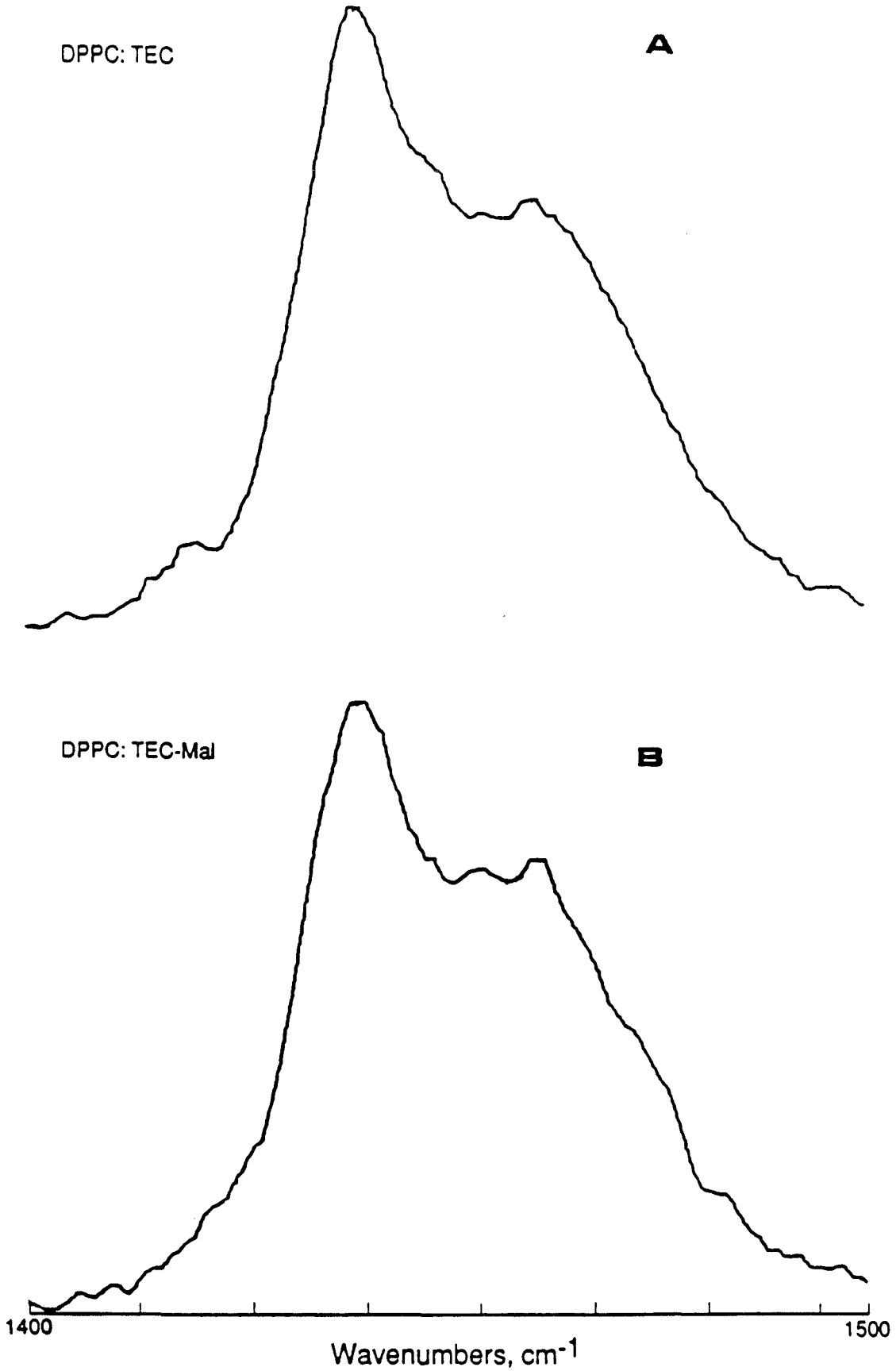
Raman spectroscopy in the acyl chain region was used to determine alterations in chain packing and interactions in the presence of the derivatives. Spectra in the 2800-3100 cm^{-1} region and the 1400-1500 cm-1 region provide information regarding lattice disorder and interchain interactions.[14] The region of 1000-1200 cm^{-1} provides information of the extent of intrachain disorder (i.e., the extent of trans/gauche isomerization).[15] Fig. 6 displays spectra in the 1400-1500 cm^{-1} region, corresponding to the methylene deformation region. In this instance, the ratio of the peak at 1460 to that at 1436 provides a measure of the extent of interchain disorder.[14] The results recorded in Table Four demonstrate that the additon of TEC or TEC-Mal results in a pronounced increase in the lattice disorder. This behavior is also exhibited in spectra accumulated in the 2800-3100 cm-1 region (Fig. 7), which provides information from C-H stretching modes. In this case, the ratio of the peak at 2937 to that at 2880 provides further evidence that the addition of TEC-Mal or TEC to the bilayer induces a decrease in interchain interactions and hence an increase in lattice disorder. As the data in Table Four indicates, there is a slight difference in this behavior from that exhibited by cholesterol. This difference is indicative of

greater expansion of the lipid lattice in the presence of derivatives bearing the carbohydrate.

Fig. 8 depicts spectra in the region of 1000-1200 cm^{-1}, corresponding to C-C stretch modes. The ratio of the peak at 1128 to that at 1062 is recorded in Table Four. This ratio decreases as the number of gauche conformers increases.[15] The results recorded here show that the intrachain disorder in samples of DPPC:TEC-Mal (7:3) is no different than that induced by the presence of equivalent amounts of TEC or CHOL in DPPC. There is no increase in the number of gauche conformers in the presence of these agents over that normally observed in pure DPPC bilayers in the dry state.

This behavior is consistent with the observation of Lee et al. that below the L_κ to L_λ phase transition, x-ray diffraction patterns are characteristic of lipid in a gel phase. Since the spectra recorded here were obtained well below the noted 49° Celsius transition, the interchain disorder observed should be consistent with that obtained for gel-phase bilayers. The results indicate that the addition of carbohydrate at the interface causes an expansion of the lipid lattice, characteristic of the expansion normally seen as lipid passes from a $P_{\beta'}$ to the L_α phase, without inducing enhanced trans/gauche isomerization below the phase transition temperature.

Fig. 6. Spectra in the 1400-1500 cm^{-1} region corresponding to methylene deformation modes. (A) DPPC:TEC (7:3), (B) DPPC:TEC-Mal (7:3). All spectra recorded at room temperature.


DPPC: TEC
A
DPPC: TEC-Mal
B
1400
1500
Wavenumbers, cm-1

Peak Ratios

Additive	1128/1062	2937/2880	1460/1436
None*	0.91	0.37,0.39**	0.95
CHOL*	0.92	0.41	0.79
TEC	0.98	0.50	0.69
TEC-Mal	0.94	0.57	0.71

Table IV: Intensity ratios in acyl chain region.

* Bush, Adams, and Levin (1980).

**This study.

Fig. 7. Spectra in the 2800-3100 cm^{-1} region corresponding to C-H stretching modes. (A) DPPC:TEC (7:3), (B) DPPC:TEC-Mal (7:3). All Spectra recorded at room temperature.

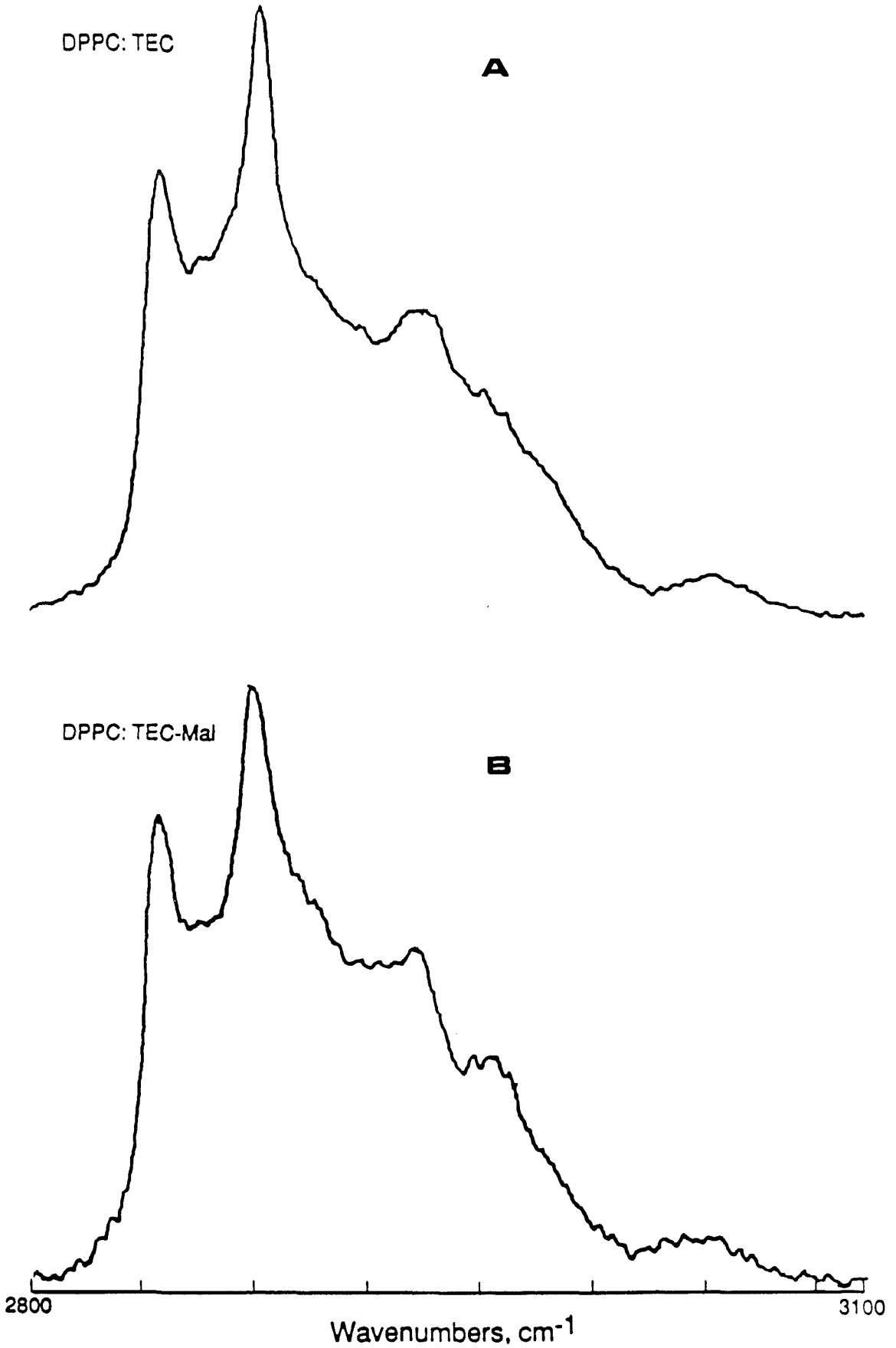
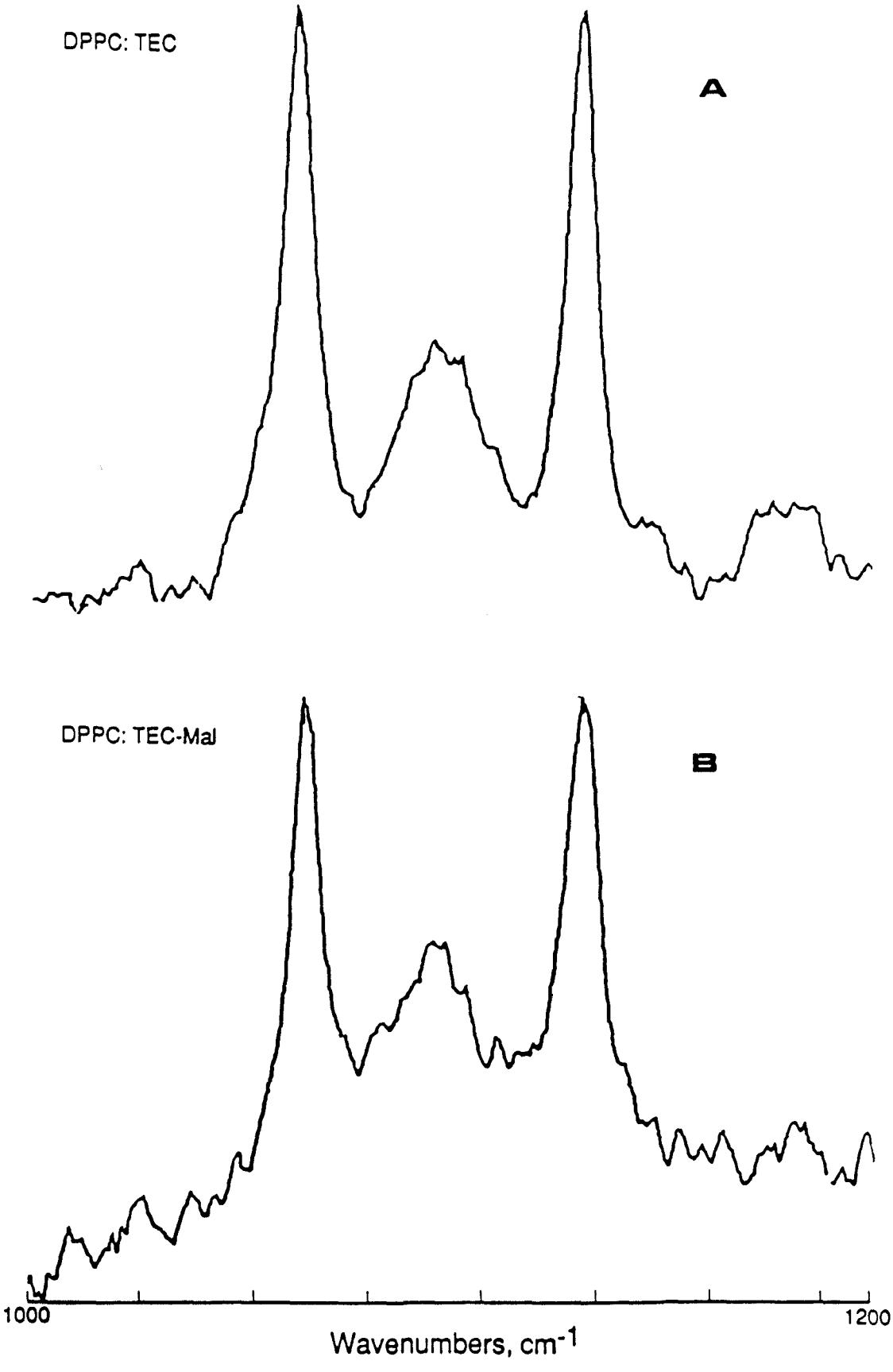

DPPC: TEC
A
DPPC: TEC-Mal
B
2800
3100
Wavenumbers, cm-1

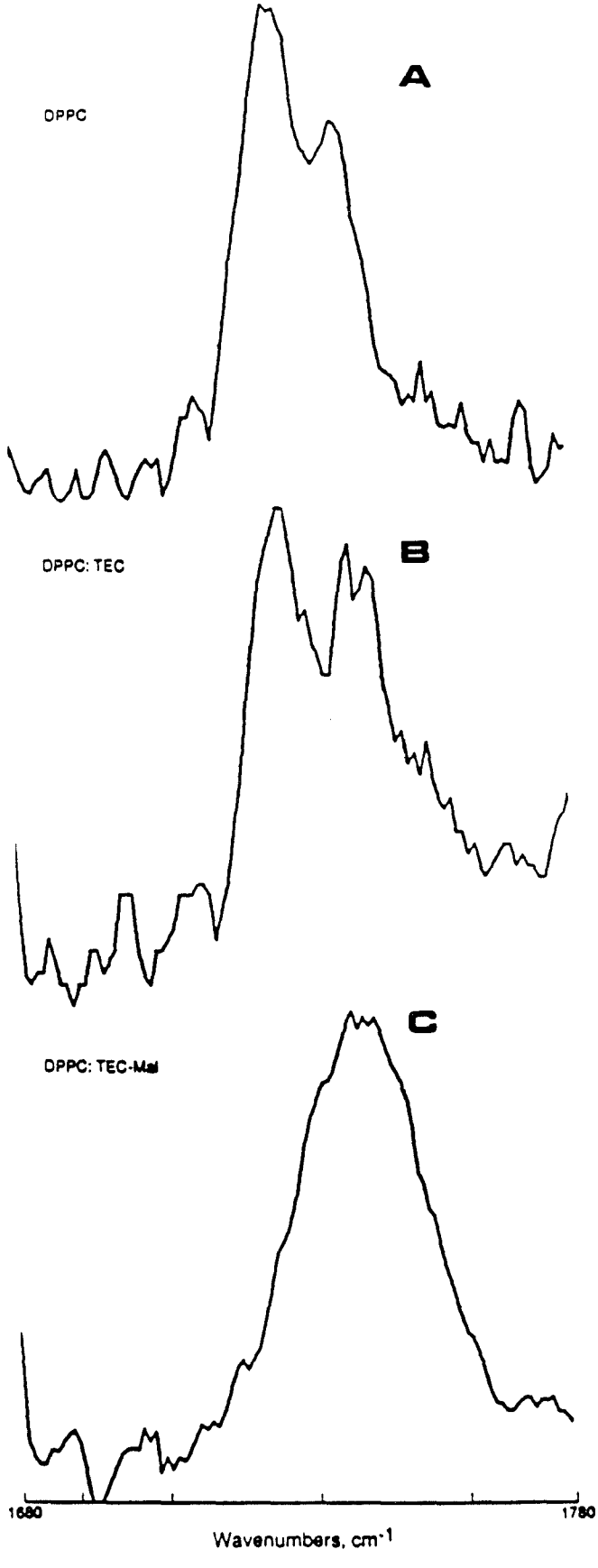
Fig. 8. Spectra in the 1000-1200 cm^{-1} region corresponding to C-C stretch modes. (A) DPPC:TEC (7:3), (B) DPPC:TEC-Mal (7:3). All spectra were recorded at room temperature.


DPPC: TEC
A
DPPC: TEC-Mal
B
1000
1200
Wavenumbers, cm-1

Raman spectroscopy was also used to probe the carbonyl region of the lipid. Bands in this region are much more intense in IR, but are often complicated due to interfering -OH bands from the carbohydrate. These transitions are not allowed in the Raman, eliminating this interference. Spectra in the 1680-1780 cm^{-1} region are depicted in Fig. 9. Dry DPPC possesses two bands at 1736 and 1726 cm^{-1}. These peaks are normally associated with the carbonyl modes of the 1 and 2- chains repectively.[16] The asymmetry of the chains is believed to be responsible for producing 2 distinct environments for the carbonyl groups. For example, the sn-2 chain carbonyl is closer to the phosphate head group, and is thus more easily affected by changes in the polar head group region.[8,17] The sn-1 chain carbonyl, however, sits in the hydrophobic region of the bilayer, and is less sensitive to such changes at the interface.[12,17] DPPC:TEC (7:3) possesses a spectrum characteristic of pure DPPC. Additional splitting is observed in this case with peaks at 1726, 1736, and 1739 cm^{-1} . The peak at 1739 cm^{-1} has been assigned to a rotational isomer of the sn-1 chain.[13] For DPPC:TEC-Mal (7:3) there is a coalescence of the two peaks into a single peak at 1740 cm^{-1}. This shift is also observed with the addition of water to dry DPPC. [12] In this case, the water hydrogen bonds to the charged phosphate group, causing an alteration of the charge distribution in the polar head group region. The additon of water also results in changes in the lipid packing, which places the sn-2 chain carbonyl closer to the hydrophobic

acyl chain region of the bilayer. Both of these changes contribute to making the sn-2 chain more electronically equivalent to the sn-1 chain and are normally associated with the transition from $L_{\beta'}$--$P_{\beta'}$--L_{α}. As noted previously, the carbohydrate moiety at the interface appears to mimic water in both of these respects. The shifts observed in the phosphate region are consistent with hydrogen bonding to this group. In addition, the carbohydrate intercalation into the interface causes an expansion of the lattice, which is conducive to reordering of the acyl chains. The observed behavior of the carbonyl group indicates that such reorganization does indeed take place. This behavior suggests that the assignment of the L_{κ} phase to a gel phase DPPC may not be entirely correct in that the hydrocarbon chains do not appear to be tilted. X-ray diffraction in the case of Lee et al. was not conclusive on this point due to the complexity of the diffraction pattern from excess crystalline trehalose. The spectra in the carbonyl region of the lipid in the presence of the carbohydrate derivative is consistent with a *trans* conformation about the carbon 2 position, a characteristic of a straight chain segment. This implies that while the acyl chain organization in the L_{κ} phase is characteristic of gel phase DPPC, the orientation of the bilayer with straight as opposed to tilted chains is more characteristic of the $P_{\beta'}$ or L_{α} phases.

Fig. 9. Spectra in the 1680-1780 cm^{-1} region corresponding to carbonyl modes. (A) DPPC, (B) DPPC:TEC, (C) DPPC:TEC-Mal. All spectra recorded at room temperature.


A
DPPC
B
DPPC: TEC
C
DPPC: TEC-Mal
1680
1780
Wavenumbers, cm-1

Conclusion

The results recorded here are consistent with postulated mechanisms for the action of carbohydrates on the bilayer. For the first time, through the use of these derivatives, a distinct stoichiometry can be obtained since excess crystalline carbohydrate is no longer a factor for consideration. In addition, the direct association of the carbohydrate with the bilayer allows us to probe the specific alterations occuring more precisely.

A distinct picture now emerges as to what actually occurs at the membrane interface when carbohydrate is added. The carbohydrate mimics the effects of water in several respects. First, there is an expansion of the lipid lattice normally associated with lipid passing from the pre-transition to a liquid crystalline state. This results in a reduction in the interchain interactions and hence a greater interchain disorder. The intrachain disorder is maintained, however, characteristic of gel phase lipid. This phase state is designated as the L_K phase by Lee et al. As a consequence of this behavior, the lipid phase transition temperature is lowered. In the case of the derivatives, a minimum of 0.42 mole/mole of lipid is required to induce the same reduction observed for TRE:DPPC in a 2:1 mole ratio. This result suggests that the actual stoichiometry of carbohydrate at the membrane surface needed to induce the modifications is actually much less than that which is present in preparations involving non-tethered carbohydrates. The presence of excess crystalline trehalose in preparations by Lee et

al. confirms that at least some of the trehalose in these samples is not interacting directly with the bilayer. It is reasonable to assume that the carbohydrate directly intercalated into the lipid interfacial region is responsible for the effects noted in their case as well because direct incorporation via the methods reported here produces the same effect without the presence of an excess carbohydrate phase.

The carbohydrate at the interface induces not only an expansion of the lipid lattice as evidenced by changes in Raman spectra in the C-N, C-C, and C-H regions, but also induces changes in the organization of the membrane. This is evidenced by alterations in the carbonyl groups. Expansion of the lipid lattice and hydrogen bonding of the carbohydrate to the lipid phosphate groups alters the interface in a manner that favors reorganization of the acyl chains into a straight chain conformation. The L_K phase is thus characterized by gel phase packing without the tilt characteristic of the gel phase. This alteration in chain packing moves the sn-2 chain carbonyl into an environment that is more hydrophobic and motionally restricted. Such alterations would also be expected to result in changes in the behavior of the motional freedom of this group, a result that has recently been obtained from ^2H NMR studies.[10]

The use of the derivatives with maximum linker group extension of approximately 10.5 Angstroms also places the carbohydrate group in a region between the lipid molecules in the hydrophilic portion of the lipid head group. This positioning is consistent with

that proposed by Lee et al. and suggests that free carbohydrates also do not merely reside at positions on the surface of a membrane because it is intercalation of the carbohydrate that produces the effects noted here. The actual extent to which the carbohydrate is intercalated and how much it resides at the bilayer surface is, however, not yet clearly defined and must be determined in part by the actual geometry of the linker group to the bilayer.

Finally, the relationship to the capacity of carbohydrates to induce these effects and their ability to protect the membrane against damage during dessication has been correlated in many instances. It is interesting to note therefore that addition of the derivative possessing all functionalities except the carbohydrate (TEC) fails to induce the full range of alterations noted here. In keeping with the proposed correlation, this compound is also ineffective at protecting SUV's from damage during freeze thawing and freeze drying.[18]

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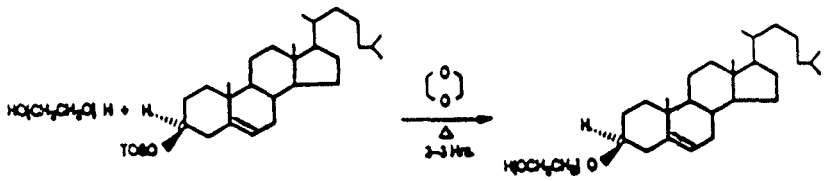
APPENDIX: SYNTHESIS AND CHARACTERIZATION OF CARBOHYDRATE DERIVATIVES

The derivatives utilized in the studies described in the preceding sections were prepared according to standard synthetic procedures outlined here (Fig. 1). The polyoxyethylene derivatives of cholesterol were prepared according to methods described previously. [1,2] The reaction is also depicted in scheme 1.



As Logani demonstrated, the reaction proceeds with retention of stereochemistry at the cholesterol 3-position.

The synthetic methodology employed for the preparation of the glycolipids permits variation in the structure of the carbohydrate moiety, configuration at the anomeric center of the carbohydrate, and length of the spacer unit. The synthesis utilizes a variation of the Koenigs-Knorr reaction which is outlined in scheme 2. [3]

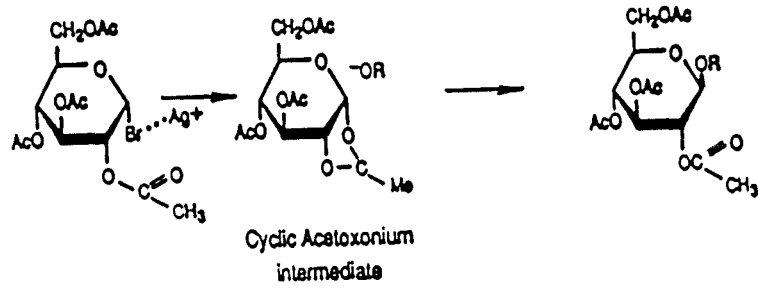
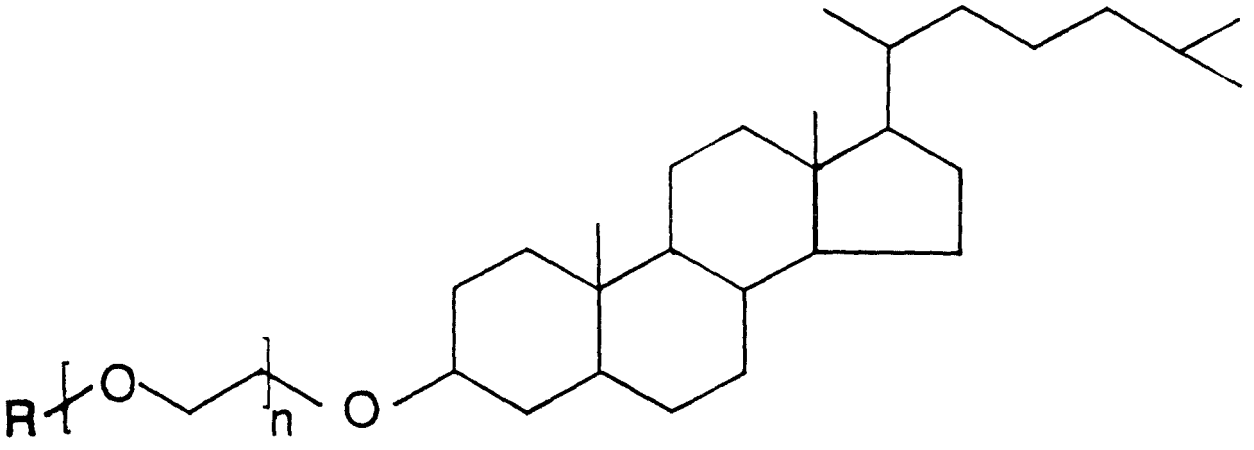
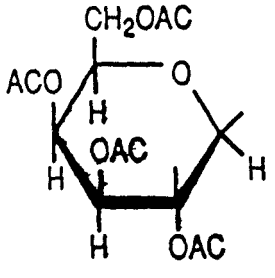
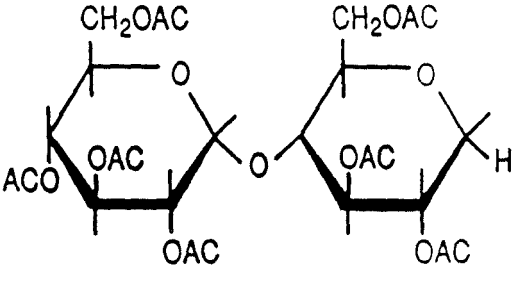


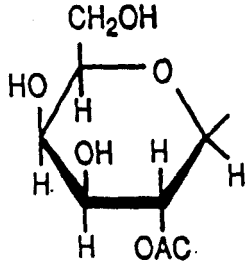
Fig. 1. Structure of synthetic glycolipids and precursors.

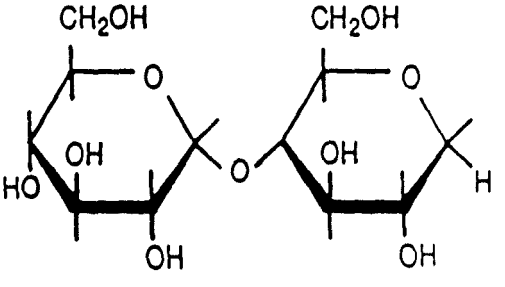


1, n = 1 R = H
2, n = 2 R = H
3, n = 3 R = H

a, R = 

c, R = 

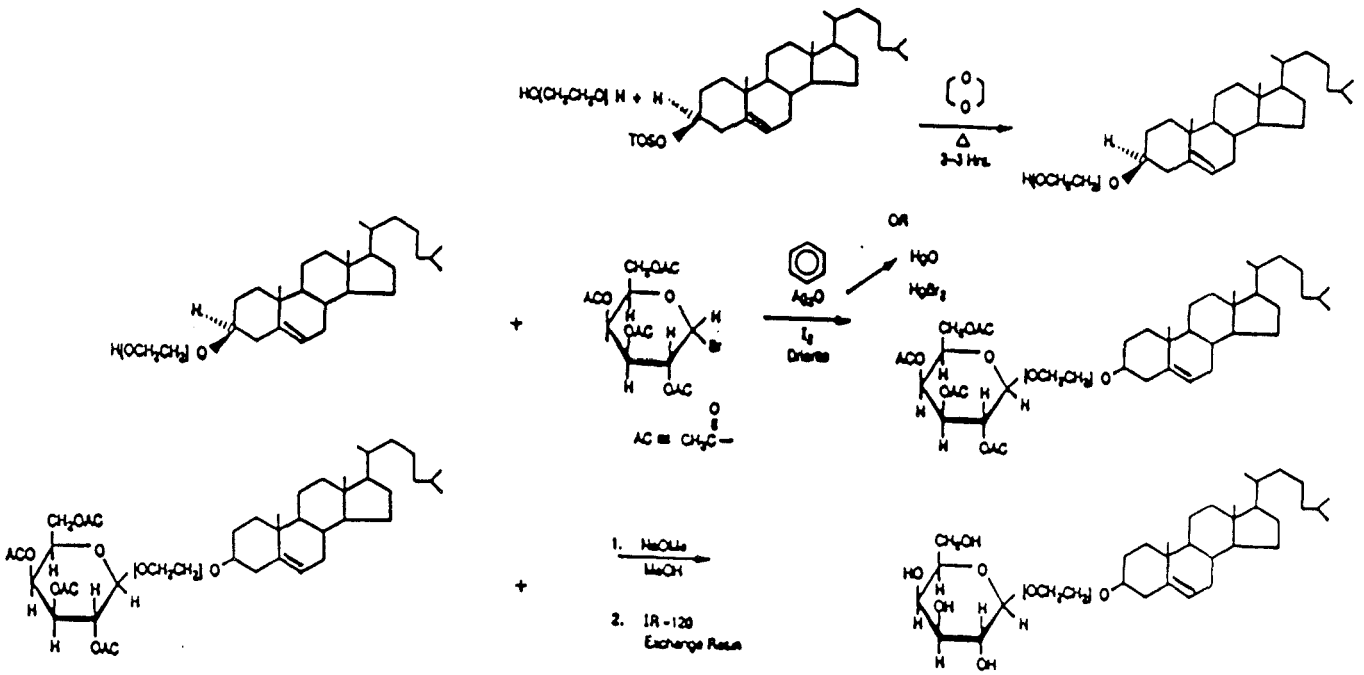
b, R = 

d, R = 

This reaction proceeds specifically via an acetoxonium ion to yield the 1,2 *trans* product as depicted in Fig. 2. [4] In the reaction, both silver as well as mercury salts may be employed.[5] As noted in Table 1, the use of mercury salts resulted in enhanced reaction yields.

Samples were characterized by thin layer chromatography (TLC) and melting point determinations. Results are summarized in Table 1. TLC was carried out on silica gel plates. The plates were treated with stain reagents to visualize the materials on the plates. The stain reagents included α-Naphthol and $FeCl_3{}^{o}6H_20$. The α-Naphthol reagent reacts specifically with carbohydrates to produce a purple color. [6] The iron chloride reacts with cholesterol, its ethers, and esters, to yield a red color. [7] Each glycolipid tested was positive with each of these reagents, yielding a single, homogeneous spot with the recorded R_f values. TLC plates were also developed using the non-specific H_2SO_4 and Haines-Isherwood reagents to visualize any additional materials on the plates. [8,9]

Fig. 2. Full reaction scheme for synthesis of glycolipid.



Compound	%Yield	R_f	Melting Point
1	64	0.54*	102-104
1a	22	0.68*	84-85
1b	23	0.62**	155-156 (d)
2	14	0.36***	59-60
2a	32	0.54***	95-97
2b	62	0.66**	193-194 (d)
3	55	0.29****	-
3a	10,26#	0.55****	-
3b	45,60#	0.68**	170-171 (d)
3c	30#	0.58****	--
3d	58#	0.36**	--

Table I: Physical Constants
*=chloroform:acetone:cyclohexane (180:20:30)
**=chloroform:methanol (80:20)
***=cyclohexane:diethyl ether (10:60)
****=chloroform:ethyl acetate (50:50)

#=Yield when Hg salts are substituted for Ag salts

(d)=Decomposes

Melting points are recorded for each product in Table 2. Each glycolipid (1b, 2b, 3b and d) decomposed at the recorded temperatures. The values recorded represent the end points for the decomposition. Crystallization of the glycolipids was difficult, resulting primarily in the formation of amorphous solid material. All products were purified by column chromotography on silica gel and stored at -22°C under argon.

Compounds were examined using high field proton NMR. The results are summarized in Table 2. The acetate protected sugars (1a,2a,3a and c) were characterized by the presence of peaks from 1.9-2.05 ppm due to the acetate groups (Fig. 3). These peaks are absent in the deprotected sugars. A first order analysis is possible for sugar protons in the acetate protected glycolipids (1a, 2a,3a and 3c). The spectra of the corresponding deprotected sugars is, however, more complex, with most sugar protons appearing around 3.0 ppm. (Fig. 4). Only the proton at the anomeric center is reported for these compounds.

Each material exhibits the peaks characteristic of the cholesterol moiety (Fig. 5). The peak at about 5.3 ppm in each of the derivatives is due to the proton at the C-3 position of the sterol (see Fig. 4). The peak at about 3.1 ppm in each derivative is normally associated with the proton at the double bond position (C-6). The presence of this peak indicates that the sterol moiety remains unsaturated at this position. Other positions normally associated with the sterol moiety are recorded in Table 3 and depicted in Fig. 5.

Compound	Peak Position (δ scale, ppm) 1a	1b	2a	2b	3a	3b
Sugar Protons						
H-1	4.58	4.25	4.58	4.27	4.58	4.27
H-2	5.19		5.20		5.20	
H-3	4.98		5.00		5.00	
H-4	5.38		5.38		5.38	
H-5	4.13		4.15		4.15	
$H\text{-}6_1$	3.62		3.67		3.67	
$H\text{-}6_2$	3.70		3.90		3.90	
Acetate Groups						
	2.01		1.98		1.94	
	2.05		2.01		2.01	
	2.07		2.05		2.05	
	2.15		2.18		2.13	

Compound	1	1a	1b	2	2a	2b	3	3a	3b
Sterol Protons									
C-3	5.32	5.32	5.30	5.24	5.35	5.27	5.30	5.34	5.27
C-6	3.14	3.14	3.15	3.19	3.19	3.19	3.13	3.16	3.19
C-18	0.58	0.67	0.67	0.63	0.63	0.63	0.56	0.63	0.63
C-19	0.94	0.98	0.96	0.97	0.98	0.97	0.93	0.98	0.97
C-21	0.84	0.90	0.92	0.90	0.90	0.90	0.79	0.90	0.90
C-26,27	0.79	0.84	0.85	0.82	0.82	0.82	0.75	0.82	0.82
Linker Protons	3.54	3.62	3.63	3.54	3.60	3.62	3.56	3.60	3.60
	3.67			3.59			3.66		

Table II: ^1H NMR Data
Spectra at 500 MHz in chloroform-d
Positions relative to chloroform peak, 7.259 ppm

Fig. 3. Proton NMR of TEC-Gal-Acetate (3a). The acetate groups appear in the region of 2.0 ppm.

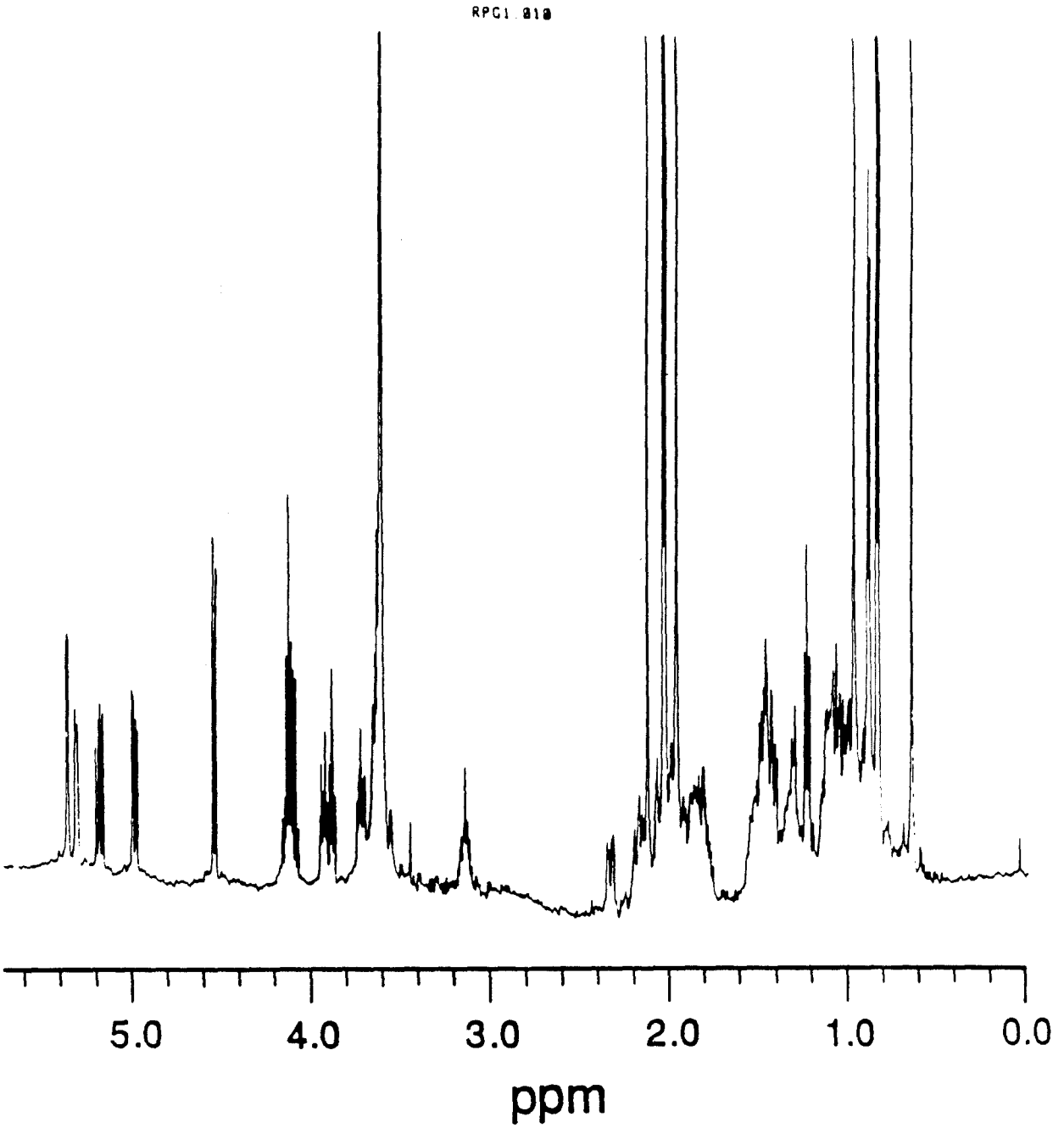

RPG1.010
TEMP.001
DATE 4-6-86
SOLVENT CDCl3
5.0
4.0
3.0
2.0
1.0
0.0
ppm

Fig. 4. Proton NMR of sugar region.

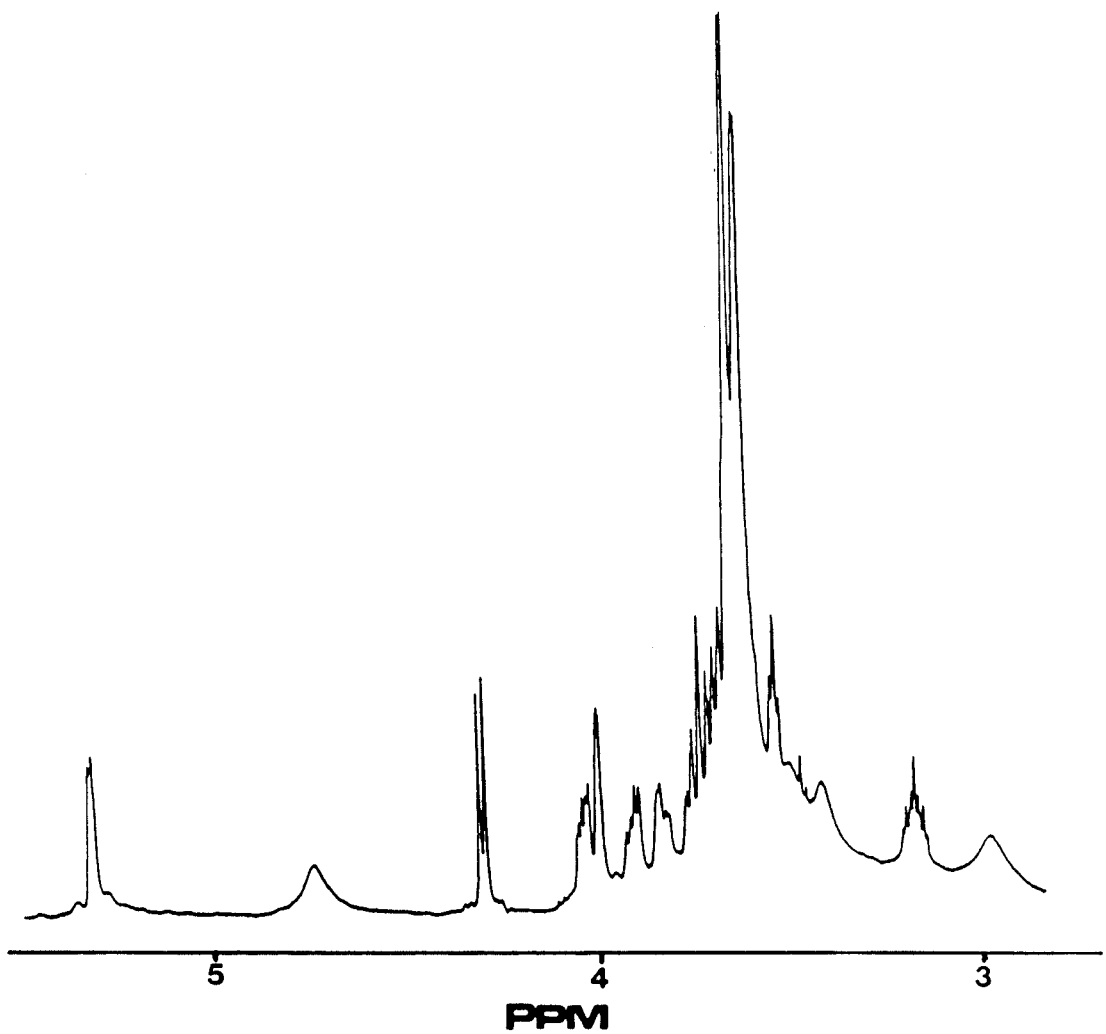
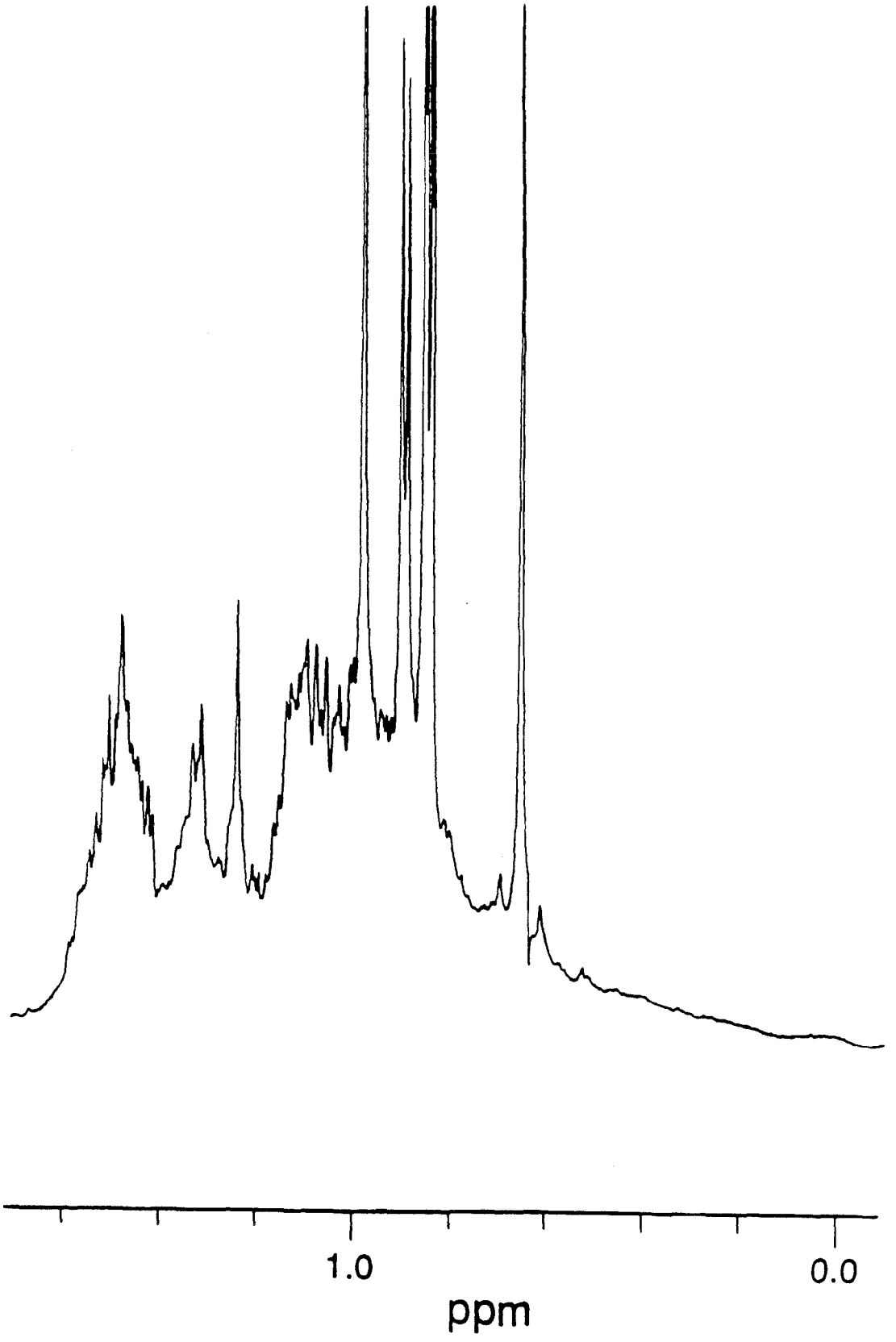

5
4
3
PPM

Fig. 5. Proton NMR of sterol protons.


1.0
0.0
ppm

Compound	Solvent	Position	$J_{1,2}$
1a	chloroform-d	4.58 ppm	7.92Hz
1b	DMSO-d6	4.25 ppm	6.0 Hz
2a	chloroform-d	4.58 ppm	8.24 Hz
2b	chloroform-d	4.27 ppm	7.59 Hz
3a	chloroform-d	4.58 ppm	8.18 Hz
3b	chloroform-d	4.27 ppm	7.01 Hz
D-(+)-Galactose	DMSO-d6	α5.9 ppm	4.5 Hz
		β4.1 ppm	6.0 Hz
Acetobromo-α-D galactose	chloroform-d	6.6 ppm	3.85 ppm

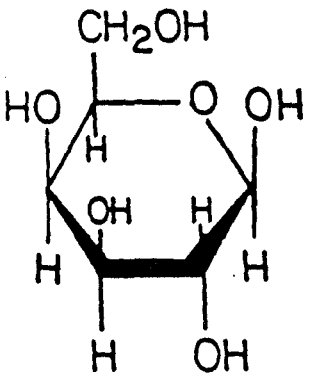
Table III: Anomeric Protons

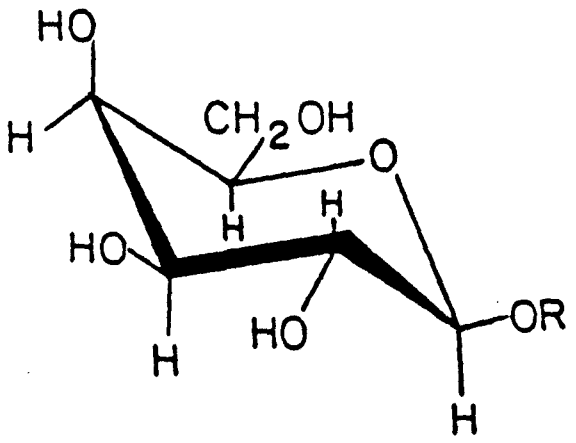
Table 3 contains coupling constants for the anomeric protons of the glycolipids, their acetates, D-(+)-galactose, and Acetobromo-α-D-galactose. The preferred conformation for galactose is depicted in Fig. 6. This corresponds to the $^4C_1$ chair conformation. As depicted, the protons at carbons 1 and 2 should exist in axial positions. The $J_{axial, axial}$ coupling constant is 8-11 Hz whereas the $J_{axial, axial}$ coupling constant is approximately 3-4 Hz. [10] Each glycolipid and its acetate exhibits a coupling constant characteristic of an axial,axial arrangement (7-8 Hz). Acetobromo-α-D-galactose exhibits that of an axial, equatorial arrangement as expected (4 Hz). The proton at the anomeric center in the acetobromo sugar occurs at such low field due to the presence of the deshielding bromine atom at position 1. D-(+)-galactose yields two peaks with two distinct values (4 Hz and 6 Hz) due to the mutarotation of the free carbohydrate in solution. The peak positions recorded for the anomeric protons in each case are also characteristic with the α derivative, giving rise to a peak at <5 ppm and the B form producing a peak at >5 ppm.

The mass spectrum of each compound is characteristic of the presence of the appropriate functional groups. These groups are recorded in Table 4 and with respect to position and intensity while the corresponding structures are depicted in Fig. 7. The peaks at 368,353,329,255,247, and 275 are indicative of the degradation of the steroid backbone.[11] For the acetate protected sugars, the peaks at 331,169, and 109 indicate the presence of the carbohydrate

moiety in a pyranose conformation.[12] In the deprotected glycolipids, peaks at 180,162,143, and 124 are indicative of the presence of a pyranose form of the carbohydrate.[13] The noted decomposition patterns are thus consistent with the presence of carbohydrate and sterol moieties in both the acetate protected and deprotected derivatives.

Fig. 6. Conformation of galactose.





$^4C_1$

Compound	m/z (rel. intensity)
1 *	430 (4), 368 (100), 329(8), 275(4) 255(7), 247(9)
	448(100),386(11)369(69),353(4) 329(4),255(4),247(5)
1a	778(91),386(11),369(100), 331(37),255(5),247(8),169(5), 157(2),115(2),109(7),95(10), 81(10),73(12)
1b	610(10),448(24),410(23),386(21) 369(100),353(2),255(2),247(3), 180(25),162(10),143(14),127(6), 124(27),113(21),95(10)
2	492(20),386(10),369(100)353(4), 255(3),247(5),124(10)
2a	822(53),454(73),438(20),410(21) 396(23),394(23),386(10), 369(100),353(6),331(89),306(15), 289(20),273(24),247(9),171(12), 169(11),157(10),145(12),124(28), 109(16)
2b	654(14),492(7),386(12),369(100), 247(5),180(26),162(15),145(16), 124(35)

Table IV: Mass Spectra Ions

3	536(11),386(4),369(100),353(4), 255(4),247(5),168(10),151(18)
3a**	871(7),369(100),331(37),247(10) 169(59)
3b	698(22),536(7),369(21),302(23), 275(15),212(100),193(12), 180(52),168(38),162(24)

Table IV: Mass Spectra Ions (continued)
*=Electron ionization
**=FAB (m-Nitrobenzyl alcohol matrix)
All other spectra were run with chemical ionization using ammonia as the reagent gas.

Fig. 7. Structures of key mass spectra ions and radicals formed at the noted molecular weights.

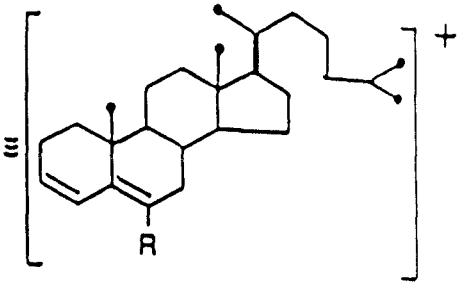
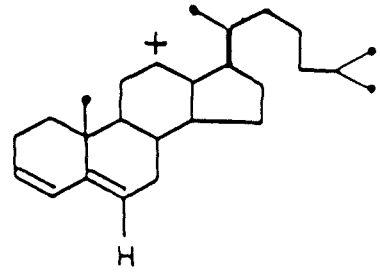
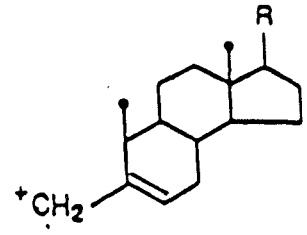
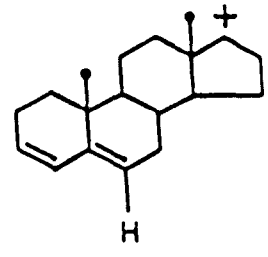
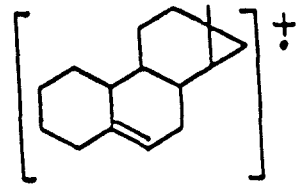
M		
430	M+	For MEC
368	$M-HOCH_2CH_2O\equiv$	
353		
329		
255		
247		

Table 5 provides high resolution exact mass data on the compounds. Electron impact ionization failed to produce a parent peak in nearly every case. As a resort, chemical ionization with ammonia reagent gas was used. The formation of a stable complex was observed for each compound.[14] Exact mass values were calculated for these peaks.

Infrared and ultraviolet spectra indicate the presence of the appropriate functionalities. Table 6 summarizes the infrared data. Compound 1a exhibits a characteristic band at 1720 cm^{-1} due to the presence of the carbonyl groups of the acetates. This band is absent in all the deacetalated glycolipid derivatives. (Fig. 8) Table 7 summarizes data obtained for ultraviolet spectra of the derivatives and trimethylethylene, a compound used as a reference chromophore.[15] The values are consistent with the presence of a single chromophore, the isolated, tri-substituted double bond of cholesterol.

Compound	m/z obs.	m/z expected	Deviation
1*	430.3797	430.3811 M+	-2.3 ppm
1a	778.5105	778.5095 MNH_4+	-1.3 ppm
1b**	631.3983	631.3976 MK+	+1.1 ppm
2	492.4417	492.4408 MNH_4+	-1.8 ppm
2a	822.5368	822.5400 MNH_4+	+3.9 ppm
2b	654.4945	654.4928 MNH_4+	-2.3 ppm
3	536.4679	536.4662 MNH_4+	-3.2 ppm
3b	698.5221	698.5207 MNH_4+	-2.0 ppm

Table V: Exact Mass Data

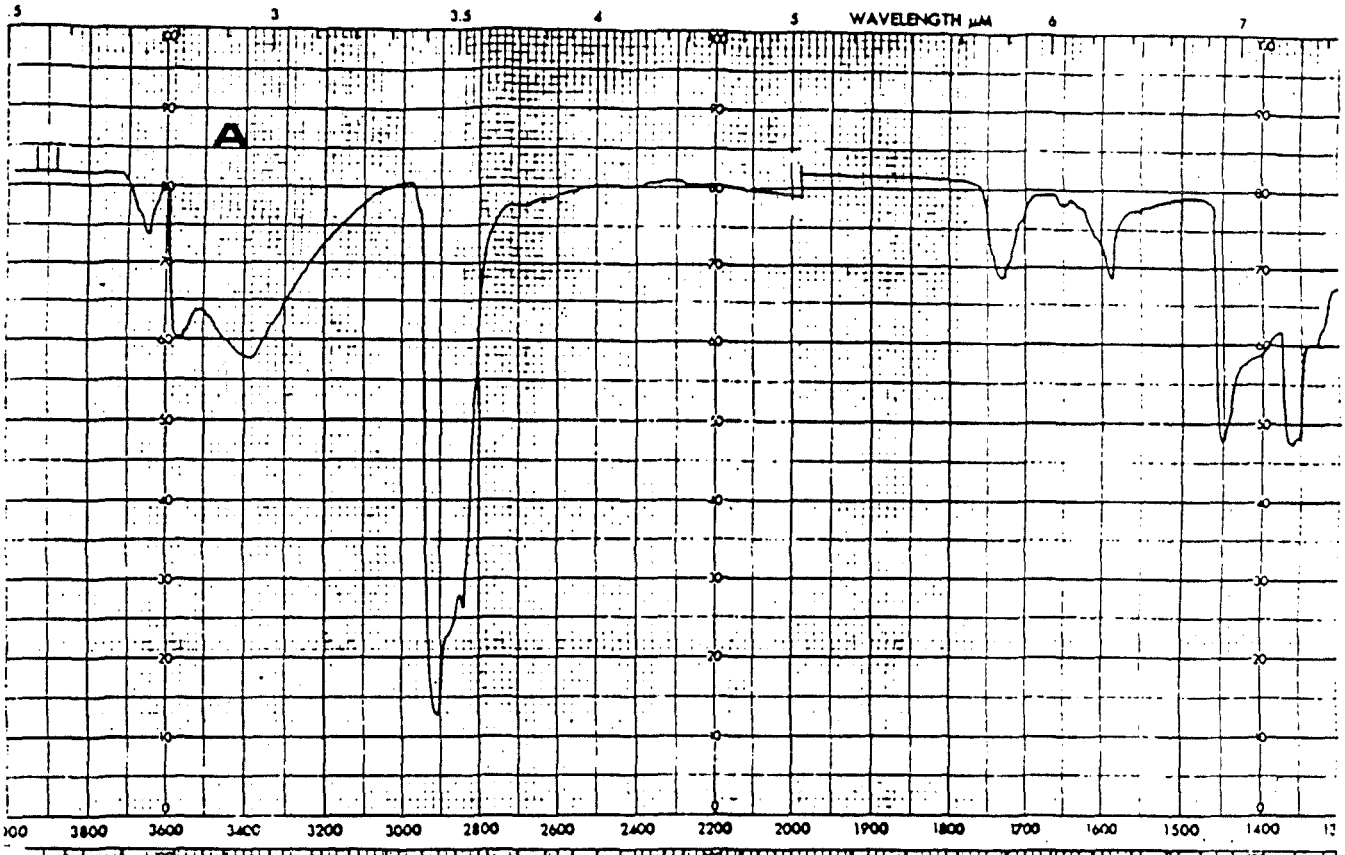
*=electron ionization
**=FAB with m-Nitrobenzyl alcohol

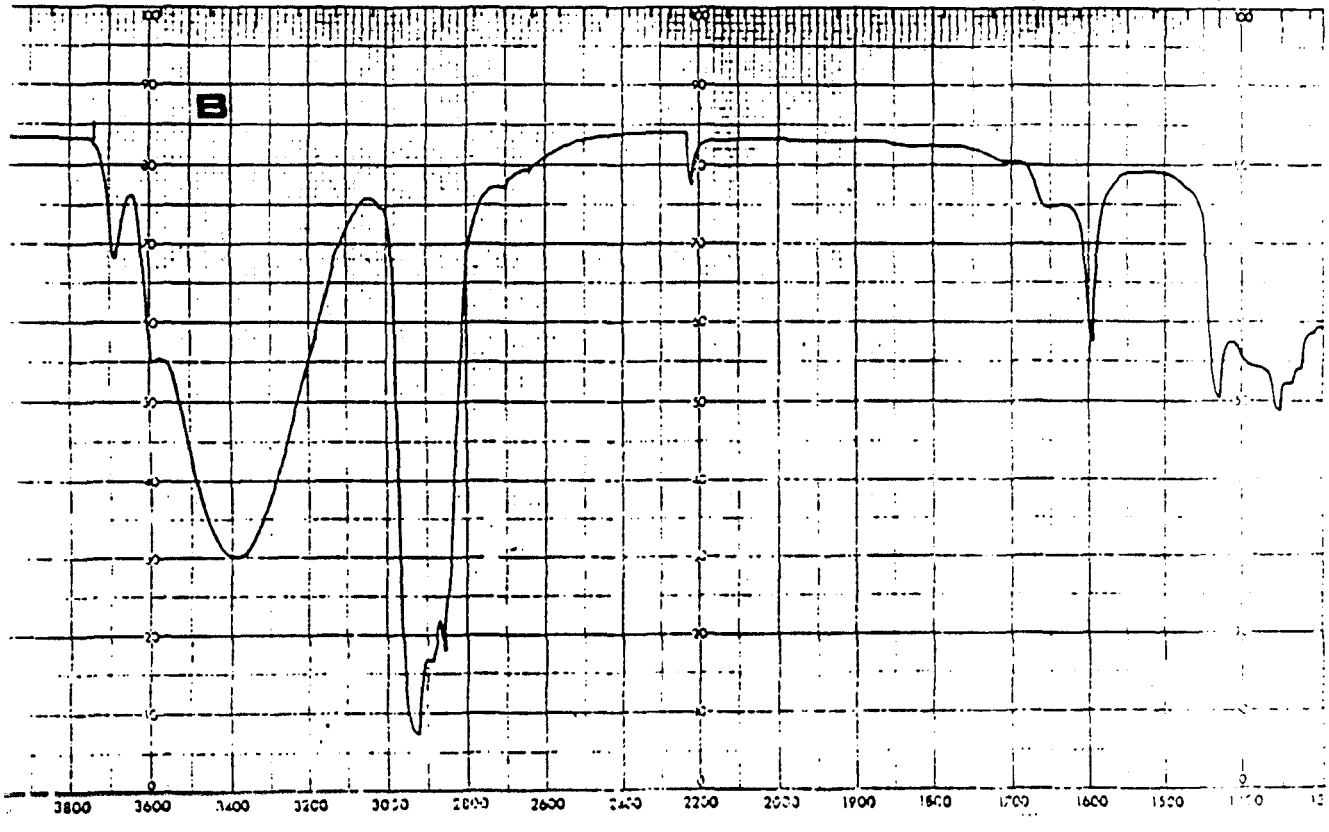
All other determinations were made from CI using NH_3 reagent gas. The predicted values represent those for stabilized ammonia complex.

Compound		
1a	3110-3610	(-OH,broad and s)
	2900	($-CH_3$,CH_2,vs)
	1720	(C=0,m)
	1580	(C=C,w)
	1040	(C-0,s)
1b	3050-3640	(-OH,broad and s)
	2920	(CH_3,CH_2,vs)
	1595	(C=C,w)
	1065	(C-O,s)
2b	3100-3620	(-OH,broad and s)
	2930	($-CH_3$, CH_2,vs)
	1600	(C=C,w)
	1025	(C-0,s)
3b	3650	(-OH,s)
	2970	($-CH_3$,CH_2,vs)
	1600	(C=C,w)
	1045	(C-0,s)
3d	3650	(-OH,s)
	2970	($-CH_3$, CH_2, vs)
	1600	(C=C,w)
	1045	(C-O,s)

Table VI: Infrared Spectra
All spectra were taken from chloroform solutions of the samples.
s=strong
m=medium
w=weak

Fig. 8. Infrared spectra of (A) Acetate protected sugar and (B) Deprotected sugar.


WAVELENGTH μM
A
3800
3600
3400
3200
3000
2800
2600
2400
2200
2000
1900
1800
1700
1600
1500
1400


B
3800
3600
3400
3200
3000
2800
2600
2400
2200
2000
1900
1800
1700
1600
1500

Compound	λmax	log ε max
1b	239	1.8
2b	239	1.6
3b	237	1.5
cholesterol	237	1.7
trimethylethylene	235	2.0*

Table VII: UV Spectra
*All spectra were recorded from solutions of the samples in chloroform except trimethylethylene, which is in hexane. (A. Luthy, Z. Physik. Chemie, 1923.)

Experimental

Preparation of Polyoxyethylene Derivatives of Cholesterol

Samples of the mono-, di-, and triethoxy derivatives of cholesterol were prepared by refluxing the appropriate polyethylene glycol with cholesteryl-ρ-toluenesulfonate in dioxane for 2-3 hours. The dioxane was removed by rotary evaporation and the resulting material taken up in water. The milky aqueous layer was extracted with ether. The organic layer was subsequently washed with a 10% aqueous sodium carbonate solution, dried over anhydrous sodium carbonate, and removed by rotary evaporation. The monoethoxy and diethoxy derivatives yielded solids whereas the triethoxy derivative formed a liquid-crystal at room temperature. All materials were purified by column chromatography on silica gel and recrystallization from methanol for the solids. Samples were characterized by TLC, NMR, mass spectra, and melting point determinations. The results are summarized in the appendix for each compound.

Preparation of Carbohydrate Derivatives

The appropriate cholesterol derivative was dissolved in benzene and placed in a round bottom flask fitted with an addition funnel. To this was added silver oxide, iodine, and powdered molecular sieves (4 Angstrom, dried at 100°C). Acetobromo-α-D-galactose, which had been dissolved in benzene, was added to the stirred mixture via the addition funnel. The sugar was added dropwise over a period of one hour. The molar ratio of silver oxide:iodine:sterol:sugar was 2:1:1:2. Alternatively, mercury oxide and mercury bromide were used in the reaction mixture in place of the silver oxide and iodine. The mixture was stirred at room temperature in the dark for 3-7 days. The progress was followed by TLC on silica gel. The mixtures were filtered and the organic solvent removed by rotary evaporation. The acetate protected glycolipids were purified by column chromatography on silica gel. Samples were characterized by TLC, NMR, mass spectra and melting points. Results are summarized in the text.

The acetate groups were removed by dissolving the compounds in methanol to which was added a small quantity of sodium methoxide (0.05M). The reaction progress was followed by TLC and litmus. The mixture was neutralized at the end of the reaction by treatment with an Amberlite exchange resin. Samples were characterized by TLC, NMR, mass spectra, ultraviolet spectroscopy, and melting points. Specific ($FeCl_3 \cdot 6H_2O$,α–Naphthol) as well as non-specific (H_2SO_4) stain reagents were used to develop TLC plates

and confirm the presence of sterol and carbohydrate moieties in the glycolipids.

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