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Alterations in Membrane Surfaces Induced by Attachment of Carbohydrates†

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ABSTRACT: We have examined the behavior of the dry phospholipid dipalmitoylphosphatidylcholine (DPPC) in the presence of several carbohydrate derivatives. These carbohydrate derivatives possess a hydrophobic portion which is incorporated directly into the DPPC membrane and a hydrophilic portion which 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 and carbonyl vibrational modes of the phospholipid portion of the membrane. In addition, the phase transition behavior of the lipid is significantly altered as evidenced by a 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 these effects by localizing the agent responsible for these alterations at the membrane interface.

We have previously described the interaction of a class of synthetic glycolipids with membrane phospholipid vesicles composed of phosphatidylcholines and phosphatidylethanolamines (Goodrich et al., 1988; Goodrich & Baldeschwieler, 1987, 1988). These compounds have also been shown to exhibit a cryoprotective and lyoprotective action (Goodrich & Baldeschwieler, 1988). This latter property is manifested in an ability to prevent lipid mixing, increase in vesicular size, and leakage of vesicular contents following freeze-thawing and freeze-drying when present in mixtures with phospholipids in a 7:3 (lipid to derivative) mole ratio. In this paper, we examine specific alterations in lipid physical properties when dipalmitoylphosphatidylcholine (DPPC)[1] is dried in the presence

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[1] Abbreviations: DPPC, dipalmitoylphosphatidylcholine; DSC, differential scanning calorimetry; FT-IR, Fourier transform infrared spectroscopy; IR, infrared spectroscopy; TRE, trehalose; TEC, triethoxycholesterol; TEC-Mal, maltosyltriethoxycholesterol; CHOL, cholesterol; L_α, liquid-crystalline lamellar phase; L_β, gel lamellar phase.

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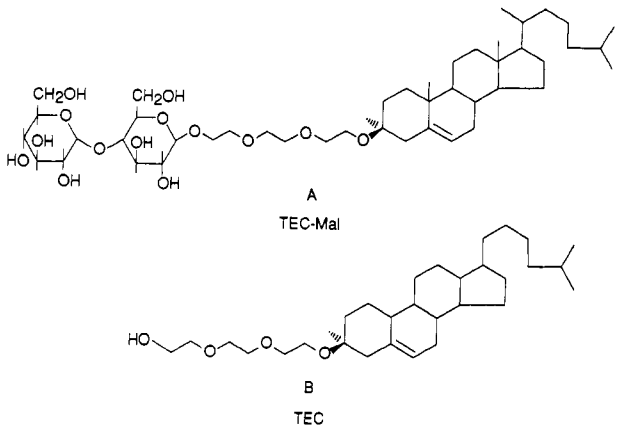

FIGURE 1: Structures of carbohydrate derivatives employed in this study. (A) TEC-Mal; (B) TEC.

of these derivatives. For this work, concentrations of derivatives in previous studies that afforded the maximum level of cryopreservation were used.

The ability of carbohydrates to interact and protect vesicles during freeze-thawing and -drying has been well documented (Strauss & Hauser, 1986; Crowe et al., 1988). This property is believed to be due to their capacity to interact directly with phospholipids in a manner which imitates the presence of water. It is this capacity which is believed to permit survival of anhydrobiotic organisms exposed to conditions of low water activity (Crowe, 1971). The interaction between carbohydrates and lipids normally manifests itself in a lowering of the gel to liquid-crystalline phase transition temperature and shifts in the phosphate asymmetric stretching mode as detected by infrared spectroscopy (Crowe et al., 1984).

Recently, Lee et al. (1986, 1989) 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. They called these new phase states the L_κ and L_λ phases, corresponding 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.

In agreement with previous suggestions, Lee et al. (1986, 1989) have postulated that the intercalation of the carbohydrate into the interfacial region induces these alterations via expansion of the lattice and modification of the whole lipid interfacial region. If this hypothesis is correct, then direct incorporation of the carbohydrate into the interfacial region should induce the same effect. In addition, as a consequence of direct incorporation into the interfacial region, the stoichiometry could be adjusted to maximize the effect without observing excess crystalline carbohydrate.

The derivative depicted in Figure 1 [previously described by Goodrich et al. (1988)] possesses a sterol backbone which resides in the acyl chain region of the bilayer. At the 3 position of the sterol, a linker polyoxyethylene chain is attached. This linker group connects the carbohydrate portion of the molecule to the sterol portion of the molecule which is incorporated into the bilayer. The length of this linker group has been preselected to provide extension away from the surface and flexibility to allow the carbohydrate moiety some freedom at the bilayer interface. It is known from X-ray crystallography and studies of specific viscosities that polyoxyethylene chains (at a polymerization degree less than 9) adapt a zigzag form under anhydrous conditions (Staudinger, 1932; Rosch, 1956). With this geometry, the length of the repeating unit in the direction of the chain axis in this case is 3.5 Å. In this study, where $n = 3$, and the system is anhydrous, the maximum theoretical length of the extension of this group away from the bilayer interface is approximately 10.5 Å. The thickness of the polar region of phosphatidylcholine dihydrates has been determined to be approximately 11 Å (Phillips et al., 1972). This would therefore place the carbohydrate moiety directly at the bilayer surface among the lipid head groups.

Materials and Methods

Lipids were purchased from Avanti Polar Lipids and used without further purification. The compounds depicted in Figure 1 were prepared according to methods described earlier (Goodrich et al., 1988). Modifications to this synthesis were performed as follows:

Synthesis of Carbohydrate Derivatives. The TEC derivative was prepared according to methods described previously (Patel et al., 1984). For synthesis of TEC-Mal, a procedure was utilized in which to a round-bottom flask was added TEC, mercury oxide, mercury bromide, and calcium sulfate in chloroform. The mixture was stirred for 30 min. 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 was 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 and then filtered. The solvent was evaporated, yielding a white solid. The compounds were examined via infrared spectroscopy, NMR, melting point analysis, mass spectroscopy, and thin-layer chromatography. 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 calorimeter or Perkin-Elmer DSC 7. Samples were dissolved in chloroform and dried for 24–48 h under vacuum. All samples were maintained under dry nitrogen throughout the process. Sample pan loading was carried out in a glovebox flushed with dry nitrogen. Scans were recorded at a rate of 30 °C/h on the Hart calorimeter or 20 °C/min on the DSC 7. Samples prepared in this manner have displayed transitions between 64 and 102 °C (Crowe et al., 1988; Chapman et al., 1967). The extent of removal of water and sample handling techniques determine the exact transition temperature which is observed. In this regard, DSC serves as a means to monitor the extent of dehydration. In these studies, DPPC, in the absence of any other compounds, consistently yielded phase transition temperatures in the range of 77 °C, a value indicative of the "dihydrate" of DPPC (Chapman et al., 1967; Quinn et al., 1988; Kodama et al., 1982).

Fourier Transform Infrared Spectroscopy. Samples for FT-IR were run on the Perkin-Elmer Model 1710 spectrometer assisted by a Perkin-Elmer 7500 data station, or Mattson Model FT-IR. Each sample was prepared by drying from chloroform under vacuum for 24–48 h. The material to be examined was placed on barium fluoride windows in a glovebox flushed with dry nitrogen. Heating and cooling were carried

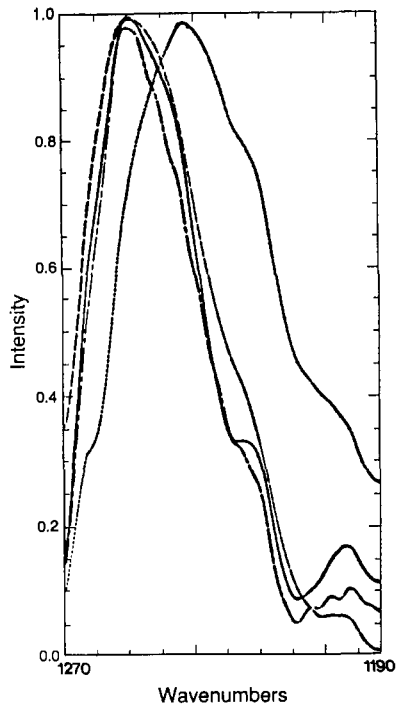

FIGURE 2: Infrared spectra in the region of the phosphate asymmetric stretching mode. Lipid:derivative mole ratios in each case were 7:3. Spectra were recorded at room temperature. (—) DPPC; (-·-) DPPC/CHOL; (---) DPPC/TEC; (···) DPPC/TEC-Mal.

out by using a Peltier device designed and constructed by Paige Instruments of Davis, CA. Temperature changes were monitored via a thermocouple attached to the sample cell holder. Approximately 50–75 transients were recorded per sample. 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 by using a 3-point method. For sample preparation, lipid was dried from chloroform under vacuum for 24–48 h. Dry material was then transferred in a glovebox 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 °C/min. 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 0.75 cm^{-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. Figure 2 depicts spectra obtained in the phosphate asymmetric stretching region for samples of dry DPPC with various additives. DPPC exhibits a phosphate asymmetric stretch at 1252 cm^{-1}. This peak remains at 1252 cm^{-1} upon addition of cholesterol or TEC (7:3 mole ratio of lipid to derivative). Upon addition of TEC-Mal, this peak is shifted to 1239 cm^{-1}. Upon heating of this sample (Figure 3), a shoulder grows into a distinct peak at 1252 cm^{-1}. Cooling of the sample leads to the disappearance of the peak at 1252 cm^{-1}.

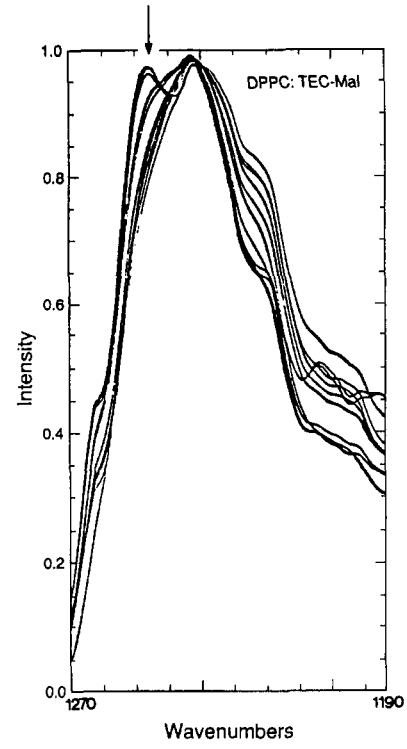

FIGURE 3: Infrared spectra in the phosphate asymmetric stretching region as a function of temperature for DPPC/TEC-Mal (7:3). Spectra were recorded over the temperature range of 22–65 °C in increments of 2–5 °C. The arrow indicates the peak at 1252 cm^{-1} that grows in intensity with increasing temperature.

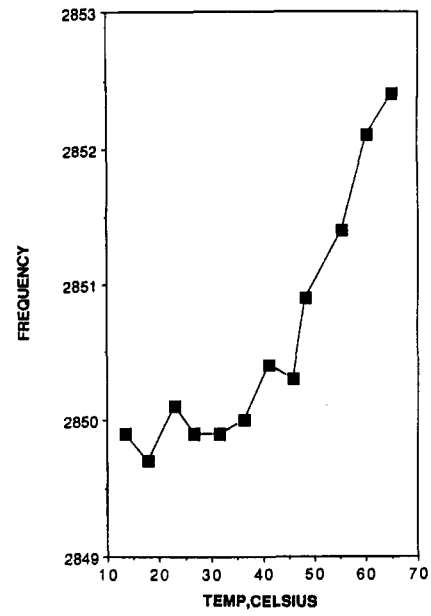

FIGURE 4: Position of the methylene asymmetric stretching mode as a function of temperature for DPPC/TEC-Mal (7:3). Data were collected from infrared spectra taken in the range of 2800–2900 cm^{-1}.

IR was also used to follow the gel to liquid-crystalline transition of the phospholipid acyl chains. Results are summarized in Figure 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). This result is also confirmed by DSC, which yields a phase

transition temperature of 51 °C for the DPPC/TEC-Mal preparation and one of 77 °C for DPPC and DPPC/TEC (7:3 mole ratio). 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 °C), as well as with the phase transition temperature observed for samples of TRE/DPPC (2:1) dried from aqueous solutions (47–53 °C) (Quinn et al., 1988; Tsetkov et al., 1989). These results would indicate that at temperatures below 51 °C, the lipid exists in the gellike phase designated as L_k by Lee et al. (1986, 1989). The induction of the L_κ phase is brought about in this case, however, by the addition of as little as 0.42 mol of the carbohydrate/mol of lipid. This represents a decrease by a factor of 4.7 in the stoichiometry. The values for T_m reported here and by Lee et al. (1986, 1989) would appear not to agree with those of Crowe et al. (1988), who reported a T_m of 24 °C for dry DPPC/trehalose mixtures. However, those results were obtained with truly anhydrous preparations, while the present one and those of Lee et al. (1986, 1989) were from the dihydrate of DPPC. These interesting differences in T_m merit further investigation.

The shifts in the phosphate region also correspond to those normally associated with the addition of water to dry DPPC (Arrondo et al., 1984). In this case, there is also a unique temperature dependence on the position of the phosphate band for samples containing the carbohydrate derivatives. The shift to lower wavenumbers of this phosphate asymmetric stretching mode upon addition of water is normally associated with the hydrogen bonding of water to the phosphate group or reorientation of this group (Mushayakarara et al., 1982; Chapman et al., 1965). 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 are 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 (Lee et al., 1986).

Raman Spectroscopy. Raman spectroscopy was used to probe the lipid interfacial and acyl chain regions. Spectra in the 680–740 cm^{-1} region are depicted in Figure 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 (Bush et al., 1980). 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 pretransition to the L_α phase. This effect has been correlated with an expansion and reorganization in the interfacial region which occurs at the pretransition (Levin, 1984).

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

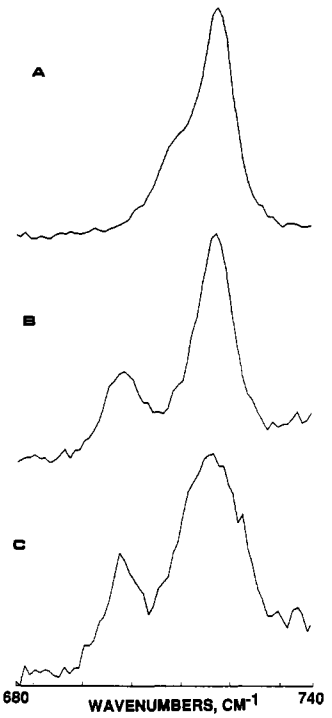

FIGURE 5: Raman spectra in the 680–740 cm^{-1} region corresponding to the C–N asymmetric stretching mode. (A) DPPC; (B) DPPC/TEC (7:3); (C) DPPC/TEC-Mal (7:3). Spectra were recorded at room temperature.

Table I: Intensity (cm^{-1}) Ratios for Peaks Obtained from Raman Spectra in the Region of Interest[a]

	peak ratios		
additive	1128/1062	2937/2880	1460/1436
none[b]	0.91	0.37,0.39[c]	0.95
CHOL[b]	0.92	0.41	0.79
TEC	0.98	0.50	0.69
TEC-Mal	0.94	0.57	0.71

[a] Changes in the ratio of 1128/1062 cm^{-1} indicate changes in intrachain disorder. *Increases* in the 2937/2880 cm^{-1} intensity ratio and *decreases* in the 1460/1436 cm^{-1} intensity ratio indicate *increases* in lattice disorder. [b] Bush et al. (1980). [c] This study.

backbone (Bush et al., 1980). This peak thus serves as an internal standard for comparing intensities in this region. 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 (ratio of 1.73) is present relative to CHOL (ratio of 2.00). The TEC derivative actually shows an increase in this ratio (2.62) when compared to CHOL. The alteration observed for 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 pretransition, an observation which is also confirmed by examination of interchain or lattice 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 (Cameron et al., 1980). The region from 1000 to 1200 cm^{-1} provides information on the extent of intrachain disorder (i.e., the extent of trans/gauche isomerization) (Gaber et al., 1978).

The results presented in Table I demonstrate that the addition of TEC or TEC-Mal results in a pronounced increase in lattice disorder. *Increases* in lattice disorder normally produce a *decrease* in the 1460 cm^{-1} component of the CH_2

deformation doublet while the intensity of the shoulder at 1436 cm^{-1} increases (Bush et al., 1980). Increases in lattice disorder can be followed by monitoring the ratio of peak intensities at 1460 and 1436 cm^{-1}. In this instance, the intensity ratio decreases in the series: no additive > CHOL > TEC, TEC-Mal. The decrease in this ratio corresponds roughly to increases in the size of the groups present at the lipid interface. This behavior is also exhibited in spectra accumulated in the 2800–3100 cm^{-1} region, which provides information from C–H stretching modes. In general, an *increase* in the intensity ratio of the peak at 2937 cm^{-1} relative to that at 2800 cm^{-1} is indicative of an *increase* in lattice disorder (Bush et al., 1980). The order of increasing intensity of this ratio follows the series no additive < CHOL < TEC < TEC-Mal. The increase in lattice disorder in these cases is beyond that explained by intercalation of the sterol moiety alone, indicating that alterations in the interfacial region also contribute to this effect.

The region from 1000 to 1200 cm^{-1} contains information due to C–C stretch modes. The ratio of the peak at 1128 cm^{-1} to that at 1062 cm^{-1} is recorded in Table I. This ratio decreases as the number of gauche conformers increases (Gaber et al., 1978). 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 DPPC in the dry state.

This behavior is consistent with the observation of Lee et al. (1986, 1989) 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 50 °C transition for DPPC/TEC-Mal (7:3 mole ratio) mixtures, the interchain disorder observed should be consistent with that obtained for gel phase bilayers. The results indicate that the addition of bulky moieties, such as the poly(ethylene glycol) and carbohydrate groups, to 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. This expansion occurs without inducing enhanced trans/gauche isomerization below the phase transition temperature.

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 Figure 6. The dihydrate form of DPPC possesses two bands at 1736 and 1726 cm^{-1}. These peaks are normally associated with the carbonyl modes of the one and two chains, respectively (Levin et al., 1982). The asymmetry of the chains is believed to be responsible for producing two 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 (Tsvetkov et al., 1989; Pearson & Pascher, 1979). The *sn*-1 chain carbonyl, however, sits in the hydrophobic region of the bilayer and is less sensitive to such changes at the interface (Bush et al., 1980; Pearson & Pascher, 1979). DPPC/TEC (7:3) possesses a spectrum characteristic of DPPC in the absence of any additives. 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 (Levin, 1984). 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 upon the addition of water to dry DPPC (Bush et al., 1980). 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. Addition of water also results in changes in the lipid packing which place 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 $L_{\beta'} \rightarrow P_{\beta'} \rightarrow L_\alpha$. 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. (1989) 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 are 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.

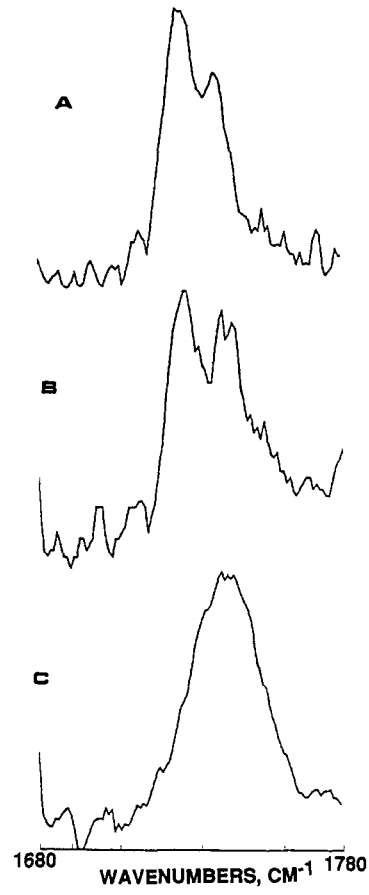

FIGURE 6: Raman spectra in the 1680–1780 cm^{-1} region corresponding to carbonyl modes. (A) DPPC; (B) DPPC/TEC; (C) DPPC/TEC-Mal. All spectra were recorded at room temperature.

CONCLUSION

The results recorded here are consistent with postulated mechanisms for the action of carbohydrates on the bilayer.

Through the use of these derivatives, a distinct stoichiometry can be obtained since excess crystalline carbohydrate is not a factor for consideration when the carbohydrate is directly incorporated into the bilayer. In addition, the direct association of the carbohydrate with the bilayer permits a more precise evaluation of the specific alterations that are occurring.

A distinct picture emerges as to what occurs at the membrane interface when carbohydrate is added in the manner described here. 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 pretransition 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_κ phase by Lee et al. (1986). As a further consequence of this behavior, the lipid phase transition temperature is lowered. In the case of the derivatives, a minimum of approximately 0.42 mol/mol of lipid is required to induce the same reduction observed for TRE/DPPC in a 2:1 mole ratio. The stoichiometry observed in this study is in close agreement with that obtained from computer modeling studies performed by Chendrasekhar and Gaber (1988) in which a TRE:DPPC ratio of 0.33:1 was obtained. The presence of excess crystalline trehalose in preparations by Lee et al. (1989) 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 since direct incorporation via the methods reported here produces the same effect without the presence of an excess carbohydrate phase.

The carbohydrate at the interface not only induces an expansion of the lipid lattice, as evidenced by changes in the 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 alter the interface in a manner which favors reorganization of the acyl chains into a straight chain conformation. The L_κ 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 which is more hydrophobic and motionally restricted. Such alteration would also be expected to result in changes in the behavior of the motional freedom of this group, a result which has recently been obtained from ^2H NMR studies (Lee et al., 1986, 1989).

The use of the derivatives with a maximum linker group extension of approximately 10.5 Å 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. (1989) and suggests that free carbohydrates also do not merely reside at positions on the surface of a membrane. The actual extent to which the carbohydrate is intercalated and how much it resides at the bilayer surface are, however, not yet clearly defined and must be determined in part by the actual geometry of the linker group to the bilayer.

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