Differential Scanning Calorimetric Study of the Interaction of Cholesterol with the Major Lipids of the *Acholeplasma laidlawii* B Membrane†

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ABSTRACT: It has been proposed that the lower levels of exogenous cholesterol incorporation into the membranes of the sterol-non-requiring as compared to the sterol-requiring mycoplasmas may be due to the much higher glycolipid content of the former and to the reduced ability of glycolipids, as opposed to phospholipids, to incorporate sterols [Efrati et al. (1986) *Arch. Biochem. Biophys. 248*, 282−288]. In order to test this hypothesis, we have investigated the interaction of cholesterol with the major membrane glyco- and phospholipids of the sterol-non-requiring mycoplasma *Acholeplasma laidlawii* B, utilizing elaidic acid-homogenous membranes in order to obviate any differences in the nature of cholesterol−lipid interactions due to variations in the fatty acid composition of the different membrane components. Specifically, we have studied the effect of increasing quantities of cholesterol on the thermotropic phase behavior of aqueous dispersions of phosphatidylglycerol, diglucosyl diacylglycerol, and monoglucosyl diacylglycerol, as well as the total membrane polar lipids of this organism, using high-sensitivity differential scanning calorimetry. We find that cholesterol is highly miscible in both the lamellar gel and liquid-crystalline states of phosphatidylglycerol but exhibits limited miscibility in the two neutral glycolipids, particularly in their lamellar gel and crystalline states. We also demonstrate that cholesterol has a limited miscibility in both the lamellar gel and liquid-crystalline states of bilayers composed of the total *A. laidlawii* B membrane polar lipids. These results demonstrate that the nature of cholesterol−lipid interactions depends markedly on the structure of the glycerolipid polar headgroup and suggests that the incorporation of lower levels of cholesterol into the membranes of the sterol-non-requiring mycoplasmas may indeed be due, at least in part, to their high glycolipid contents. We also show that cholesterol stabilizes the lamellar liquid-crystalline phase of the monoglucosyl diacylglycerol relative to the inverted hexagonal phase at all sterol concentrations, in contrast to the effects of cholesterol on dielaidoylphosphatidylethanolamine, which destabilizes the lamellar liquid-crystalline phase at low concentrations.

Acholeplasma laidlawii B is a member of the mycoplasmas, a phenotypically diverse but monophyletic group of procaryotic microorganisms that lack a cell wall [see Maniloff (1992)]. The mycoplasmas are genetically and morphologically the simplest organisms capable of autonomous replication and they thus provide useful minimal models for the study of a number of problems in molecular and cellular biology. Mycoplasmas are particularly valuable for studies of the structure and function of cell membranes. Being nonphotosynthetic procaryotes as well as lacking a cell wall or "outer membrane", mycoplasma cells possess only a single limiting membrane. This membrane contains essentially all the cellular lipid and, because these cells are small, a substantial fraction of the total cellular protein as well. Due to the absence of a cell wall, substantial quantities of highly pure membranes can be easily prepared by gentle osmotic lysis followed by differential centrifugation, a practical advantage not offered by other procaryotic microorganisms [see McElhaney (1992a)].

Another useful property of mycoplasmas in general, and of *A. laidlawii* in particular, is the ability to induce dramatic yet controlled variations in the composition of their membrane lipids. Thus, relatively large quantities of a number of exogenous saturated, unsaturated, branched chain, or alicyclic fatty acids can be biosynthetically incorporated into the membrane phospho- and glycolipids of these organisms. In cases where *de novo* fatty acid biosynthesis is either inhibited or absent, fatty acid-homogeneous membranes (membranes whose lipids contain only a single species of fatty acyl chain) can be produced. Moreover, by growing mycoplasmas in the presence or absence of various quantities of cholesterol or other sterols, the amount of these compounds present in the membrane can be dramatically altered. As well, other manipulations of the growth medium can produce large changes in the types and quantities of the various membrane lipids synthesized by these organisms. The ability to manipulate membrane lipid fatty acid and polar headgroup composition and membrane cholesterol content, and thus to alter the phase state, fluidity, and surface properties of the membrane lipid bilayer, makes these organisms ideal for studying the roles of lipids in biological membranes. For these reasons, we probably know more about the structural and functional roles of lipids in the *A. laidlawii* membrane than in any other biological membrane system [see McElhaney (1992a−c)].

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Cholesterol and related sterols are generally found only in eucaryotes, where they play an essential structural and functional role, particularly in the plasma membrane, and they may serve other specific metabolic and regulatory functions as well [see Nes (1973), Nes and McKean (1977), Yeagle (1988), and Dahl and Dahl (1988)]. However, many mycoplasma species are unique among the procaryotes in also having an absolute growth requirement for cholesterol or a closely related sterol. Specifically, members of the mycoplasma genera *Anaeroplasma*, *Mycoplasma*, *Spiroplasma*, and *Ureaplasma* require exogenous cholesterol for growth and incorporate substantial quantities of this or a closely related sterol into their cell membranes. In contrast, members of the mycoplasma genera *Asteroleplasma* and *Acholeplasma* do not require cholesterol for growth but can incorporate varying but moderate amounts of sterols into their cell membranes. The specificity of the sterol requirement for growth in the former genera, and the mechanism of sterol incorporation and the effects of sterol incorporation of membrane organization and function in both the sterol-requiring and sterol-non-requiring mycoplasma species, have been extensively studied, particularly in *A. laidlawii* [see McElhaney (1992a−c)]. Thus it has been demonstrated, using low-sensitivity DSC,[1] that incorporation of moderate amounts of cholesterol into the *A. laidlawii* membrane decreases the temperature, enthalpy, and cooperativity of the lipid gel to liquid-crystalline phase transition (de Kruijff *et al*., 1972, 1973), and that in several *mycoplasma* species the incorporation of large amounts of cholesterol abolishes this transition entirely (Rottem *et al*., 1973; Rottem, 1981). In general, the effects of cholesterol incorporation on the organization and permeability of various mycoplasma membranes are similar to those observed in model lipid bilayer membranes, where the presence of cholesterol appears to produce a liquid-ordered state, most of whose properties are intermediate between the gel and liquid-crystalline states of the phospholipid bilayer in the absence of cholesterol [see Vist and Davis (1990) and McMullen and McElhaney (1996)].

The sterol-non-requiring mycoplasmas such as *A. laidlawii* generally contain large quantities of neutral glycolipids, primarily mono- and diglycosyldiacylglycerols, whereas these neutral glycolipids are much reduced or absent in the sterol-requiring mycoplasmas. Efrati *et al*. (1986) have proposed that the lower levels of exogenous cholesterol incorporation characteristic of the sterol-non-requiring mycoplasmas may be due to their elevated glycolipid content and to a reduced capacity of these glycolipids to incorporate sterols. Moreover, the relatively stronger affinity of cholesterol for phospholipids and its relatively weaker affinity for glycolipids have been invoked to explain the existence of two separate pools of cholesterol which exchange with different kinetics in *A. laidlawii* membrane/liposomal systems (Davis *et al*., 1984) and which may exist in two different physical states (Monck *et al*., 1993). In order to investigate these questions further, we studied the effect of the incorporation of increasing quantities of cholesterol on the thermotropic phase behavior of isolated *A. laidlawii* membranes, and of the major phospholipid and of the two major glycolipids thereof, using high-sensitivity DSC. Our results do indeed indicate that the nature of the interaction of cholesterol with each of the major *A. laidlawii* membrane lipids differs considerably. Specifically, we find the apparent miscibility of cholesterol with the neutral glycolipids of this organism to be lower than with the major phospholipid, lending support to the hypothesis of Efrati *et al*. (1986), and to the possibility that the relative immiscibility of cholesterol in the neutral glycolipids may account for the presence of solid-like, laterally segregated, cholesterol-enriched domains in the membranes of this organism (Monck *et al*., 1993).

MATERIALS AND METHODS

A. laidlawii B cells were grown at 37 °C in a lipid-poor growth medium and harvested at the mid-log phase of growth as described previously (Silvius & McElhaney, 1978a,b; Silvius *et al*., 1980). Avidin, an inhibitor of *de novo* fatty acid biosynthesis and exogenous fatty acid chain elongation in this organism, was added to the growth medium, as was exogenous elaidic acid. Under these conditions, the fatty acyl groups of the lipids from these "fatty acid-homogeneous membranes" essentially consist only of elaidoyl residues (>98 mol %). The polar lipids were extracted from isolated membranes and the individual lipid components purified, separated, and quantitated as already reported (Monck *et al*., 1993). The elaidic acid and cholesterol were purchased from NuChek Prep, Inc. (Elysian, MN) and Avanti Polar Lipids (Alabaster, AL), respectively, and were used as received.

For the DSC analyses, known amounts of PG, MGDG, DGDG, cholesterol, or the *A. laidlawii* total membrane lipids were dissolved in chloroform/methanol (2:1) to make stock solutions from which the lipid/cholesterol mixtures were subsequently made by mixing appropriate amounts of each solution. The binary lipid/cholesterol mixtures were then heated to approximately 30−50 °C under N_2 to remove the organic solvents and dried under vacuum overnight. The dried lipid/cholesterol mixtures were suspended in a buffer consisting of 50 mM Tris, 10 mM EDTA, and 100 mM NaCl at pH 7.4, heated to approximately 10−20 °C above the phase transition of the mixture, and then vortexed to give a multilamellar suspension. The high-sensitivity DSC thermograms of the lipid/cholesterol suspensions were recorded with a Hart high-sensitivity differential scanning calorimeter (Provo, UT). The heating and cooling scan rates used were progressively increased from 10 to 60 °C/h as the amount of cholesterol present in the sample increased. In addition, the amount of phospholipid or glycolipid used for HS-DSC analysis was progressively increased from 0.5−2.0 mg for the pure phospholipid or glycolipid to 15 mg for samples containing 50 mol % cholesterol. This protocol has been demonstrated previously to be optimal for accurately detecting the broad, low enthalpy endotherms observed at high cholesterol concentrations (McMullen *et al*., 1993), particularly in the total membrane lipid and PG samples. The Hart calorimeter was calibrated using solid standards from Hart Scientific as well as aqueous synthetic lipid samples of high purity. Sample runs were repeated at least three times to

[1] Abbreviations: DSC, differential scanning calorimetry; PG, phosphatidylglycerol; MGDG, monoglucosyldiacylglycerol; DGDG, diglucosyldiacylglycerol; APG, acyl polyprenylglucoside; GPMGDG, glycerylphosphorylmonoglucosyldiacylglycerol; GPDGDG glycerylphosphoryldiglucosyldiacylglycerol; TLC, thin-layer chromatography; L_c, lamellar subgel phase; L_β, lamellar gel phase with untilted hydrocarbon chains; L_β', lamellar gel phase with tilted hydrocarbon chains; P_β', rippled lamellar gel phase with tilted hydrocarbon chains; L_α lamellar liquid-crystalline phase; H_{II}, reversed hexagonal liquid-crystalline phase; PC, phosphatidylcholine; DEPC, dielaidoylphosphatidylcholine; PE, phosphatidylethanolamine.

Table 1: Polar Headgroup Composition of the Membrane Lipids from *A. laidlawii* B Cells Grown in the Presence of Exogenous Elaidic Acid and Avidin[a]

membrane lipid	quantity present (mol %)	membrane lipid	quantity present (mol %)
APG	1 ± 0.5	PG	27.3 ± 6.0
MGDG	43 ± 6.3	GPMGDG	0.5 ± 0.2
DGDG	20.7 ± 4.7	GPDGDG	7.2 ± 2.0

[a] Values presented are the arithmatic means (averages) and standard deviations from the mean of three independent experiments.

ensure reproducibility. This protocol provided fully reproducible thermograms. After the HS-DSC scans, the samples were checked for degradation using TLC on Silica Gel G plates with the developing solvent chloroform/methanol/water (70:25:3, by volume) (glycolipids) or chloroform/methanol/glacial acetic acid/water (60:40:10:4, by volume) (PG) and charred after spraying with sulfuric acid. The plates were overloaded to ensure that small traces of lipid degradation products would be visualized. No degradation was observed.

RESULTS

It is well established from studies of synthetic phospholipid/cholesterol binary mixtures that the detailed nature of the interaction of phospholipids with cholesterol depends on the fatty acyl group chain length and degree of unsaturation as well as on the structure of the phospholipid polar headgroup [see McMullen and McElhaney (1996)]. Thus, in order to separate the effects of variations in lipid polar headgroup structure from variations in fatty acid composition, the major membrane lipids from fatty acid-homogeneous *A. laidlawii* membranes were studied. We choose elaidic acid as the exogenous fatty acid with which to grow fatty acid-auxotropic *A. laidlawii* B cells for several reasons. First, this fatty acid produces excellent growth when *de novo* fatty acid biosynthesis and chain elongation are completely inhibited with avidin (Silvius & McElhaney, 1978a,b). Second, the phase transition temperatures of the individual elaidic acid-homogeneous membrane lipids are in a convenient temperature range (5−40 °C) for measurement by high-sensitivity DSC (Silvius *et al*., 1980; Lewis & McElhaney, 1995). Third, the total membrane lipids are exclusively in the biologically relevant liquid-crystalline state at 37 °C, the optimal growth temperature of this organism (McElhaney, 1974a,b). Fourth, the mean hydrophobic length of elaidic acid-homogeneous membrane lipids is close to that of the cholesterol molecule itself (McMullen *et al*., 1993), thus maximizing the miscibility of cholesterol with the various *A. laidlawii* membrane phospho- and glycolipids.

The polar headgroup composition of the total membrane lipids from *A. laidlawii* cells grown in the presence of exogenous elaidic acid and avidin, an inhibitor of *de novo* fatty acid biosynthesis and exogenous fatty acid chain elongation in this organism, are shown in Table 1. The three major lipid components of such elaidic acid-homogeneous membranes are the neutral glycolipids MGDG and DGDG and the anionic phospholipid PG. The glycerylphosphorylated glycolipid GPDGDG is present in much smaller quantities, while the APG and GPMGDG are quite minor lipid components in cells grown under these conditions. In this study we examined the interaction of cholesterol only

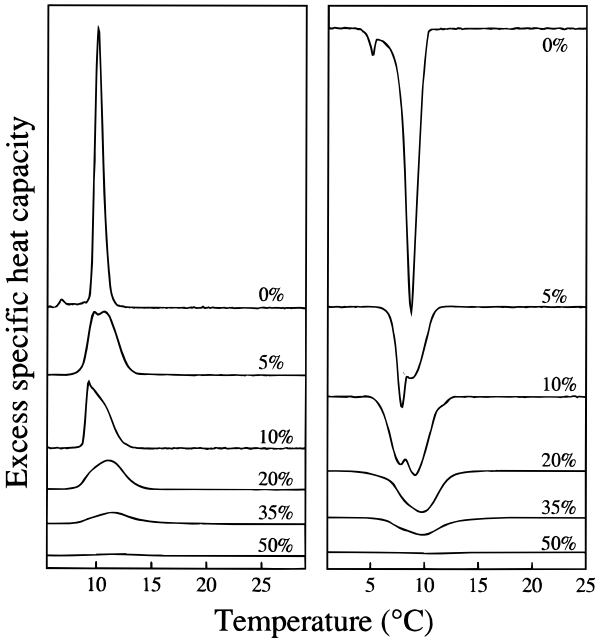

FIGURE 1: Representative DSC heating (left panel) and cooling (right panel) scans of aqueous dispersions of the PG from elaidic acid-homogeneous *A. laidlawii* membranes containing various concentrations of cholesterol.

with the three major lipids of the *A. laidlawii* membrane, since these make up over 90 mol % of the total membrane lipid. PG and DGDG are strongly and weakly lamellar phase-preferring lipids, respectively, under physiologically relevant conditions and exist only as bilayer phases, while MGDG is a strongly reversed phase-preferring lipid, existing in the lamellar phase at lower temperatures and in the H_{II} phase at higher temperatures [see Foht *et al*. (1995)].

Representative high-sensitivity DSC heating and cooling scans of the PG from elaidic acid-homogeneous *A. laidlawii* membranes, alone or in the presence of increasing quantities of cholesterol, are shown in Figure 1. PG alone exhibits a lower temperature, lower enthalpy pretransition (L_{β}'/P_{β}' transition) near 5 °C and a higher temperature, higher enthalpy main or chain-melting (P_{β}'/L_{α}) phase transition near 11 °C. The pretransition is abolished at very low concentrations of cholesterol (<5 mol %). At low concentrations of cholesterol (<20 mol %), the asymmetric DSC heating endotherms and cooling exotherms of the main transition clearly consist of two components. The phase transition temperature (Figure 2) and cooperativity of the sharp component decrease slightly with increasing cholesterol incorporation while the transition enthalpy (Figure 3) decreases markedly, becoming zero by 20 mol % cholesterol. In contrast, the phase transition temperature of the broad component of the DSC endotherms and exotherms increases slightly with increasing cholesterol concentration (Figure 2) and the cooperativity decreases markedly. The enthalpy of the broad component at first increases as a function of cholesterol incorporation up to a level of about 20 mol % and then decreases to zero at cholesterol concentrations near 50 mol % (Figure 3). The total enthalpy of the main transition thus decreases more or less linearly with increasing cholesterol incorporation (Figure 3) and a cooperative chain-melting phase transition is no longer detectable at 50 mol % cholesterol (Figure 1). The effect of the incorporation of increasing quantities of cholesterol on the thermotropic phase behavior of elaidic acid-enriched PG is essentially identical

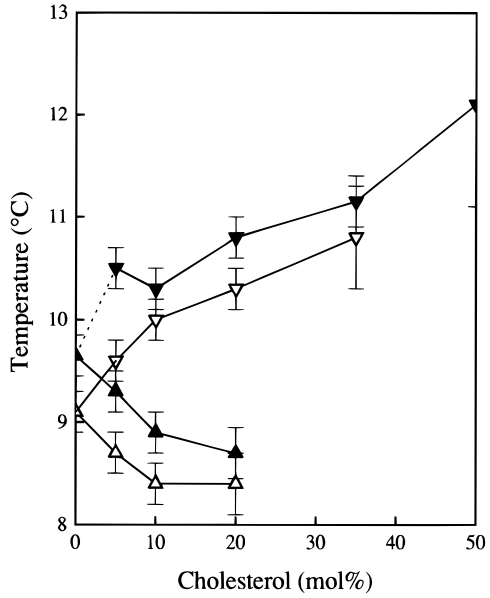

FIGURE 2: Representative plots of the temperatures of the broad (heating, ▼; cooling, ▽) and sharp (heating, ▲; cooling, △) components of the main phase transition of aqueous dispersions of the PG from elaidic acid-homogeneous *A. laidlawii* membranes as a function of cholesterol concentration. Transition temperatures were corrected for differences in heating scan rates between the various samples analyzed.

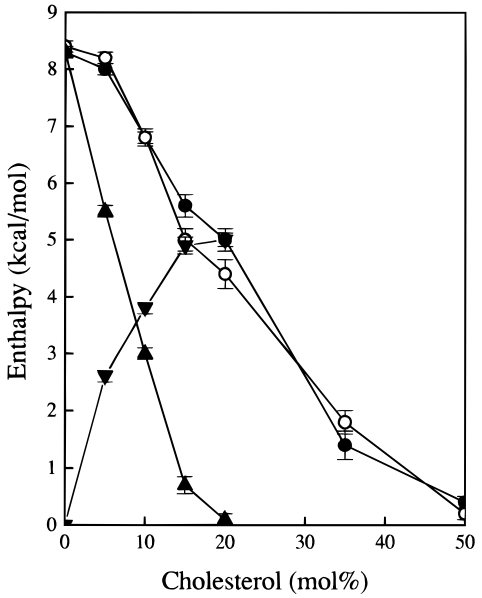

FIGURE 3: Representative plots of the total enthalpy (heating, ●; cooling, ○) and the enthalpy of the sharp (▲) and broad (▼) components, of the main phase transition of aqueous dispersions of the PG from elaidic acid-homogeneous *A. laidlawii* membranes as a function of cholesterol concentration.

to that observed for the linear saturated PCs [see McMullen *et al*. (1993)] as well as for DEPC (McMullen *et al*., 1996a). As previously, we ascribe the sharp and broad components of the DSC heating and cooling thermograms to the melting of the phospholipid hydrocarbon chains of cholesterol-poor and cholesterol-rich PG domains. Note that the effects of cholesterol on the thermotropic phase behavior of the *A. laidlawii* PG are identical in the heating and in the cooling modes, suggesting that cholesterol is fully miscible in both the gel and liquid-crystalline phases of the PG bilayer over the full range of cholesterol concentrations studied, as is also the case for PC's (McMullen *et al*., 1993, 1996a).

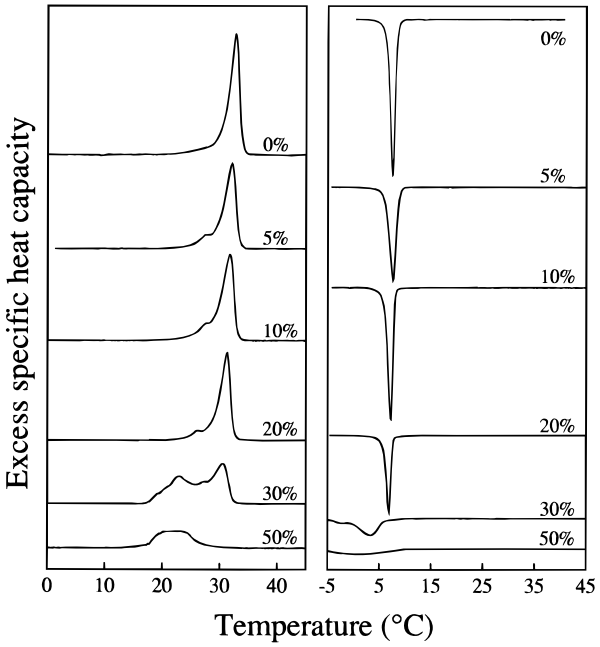

FIGURE 4: Representative DSC heating (left panel) and cooling (right panel) scans of aqueous dispersions of the DGDG from elaidic acid-homogeneous *A. laidlawii* membranes containing various concentrations of cholesterol.

Representative high-sensitivity DSC heating and cooling scans of DGDG from elaidic acid-homogeneous *A. laidlawii* membranes, alone or in the presence of increasing quantities of cholesterol, are presented in Figure 4. DGDG alone exhibits an asymmetric, moderately cooperative, highly energetic phase transition centered near 33 °C on heating, and a symmetric, highly energetic, and highly cooperative phase transition centered near 7 °C upon cooling. Both of these thermotropic events were identified as net interconversions between the L_c and L_α phases by FTIR spectroscopy (unpublished experiments from this laboratory). Although the L_β phase doubtlessly forms transiently upon cooling, the L_β phase of dieladoyl DGDG is unstable with respect to the L_c phase and rapidly converts to the latter on the time scale of the DSC experiment.

In contrast to the results obtained with PG, the DSC thermograms of elaidic acid-enriched DGDG/cholesterol mixtures containing less than 30 mol % are similar to that of pure DGDG bilayers in both the heating and cooling modes, except for a small, low-temperature shoulder on the main transition endotherm observed on heating. Upon heating, the DSC thermograms of DGDG/cholesterol mixtures containing 30 mol % or more cholesterol exhibit at least two major components. From 30 to 50 mol % cholesterol, the observed endothermic peak also broadens significantly, shifts downward in temperature (Figure 5), and its enthalpy (Figure 6) decreases sharply as the cholesterol content increases. Nevertheless, even at 50 mol % cholesterol, a broad endothermic event of appreciable enthalpy is still observed at temperatures near 22 °C. The endothermic events observed upon heating of all the DGDG/cholesterol mixtures studied were assigned to L_c/L_α phase transitions by FTIR spectroscopy (data not shown).

Upon cooling, DGDG/cholesterol mixtures containing less than 30 mol % cholesterol exhibit exotherms whose shapes are similar to those of DGDG alone. Moreover, the transition is shifted to slightly lower temperatures and moderate

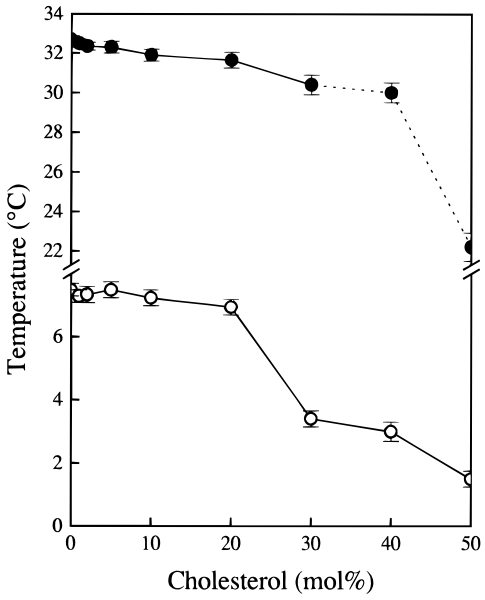

FIGURE 5: Representative plots of the temperatures of the L_c/L_α (heating, ●) and L_α/L_c (cooling ○) phase transitions of aqueous dispersions of DGDG from elaidic acid-homogeneous *A. laidlawii* membranes as a function of cholesterol concentration. Transition temperatures were corrected for differences in heating and cooling scan rates between the various samples analyzed.

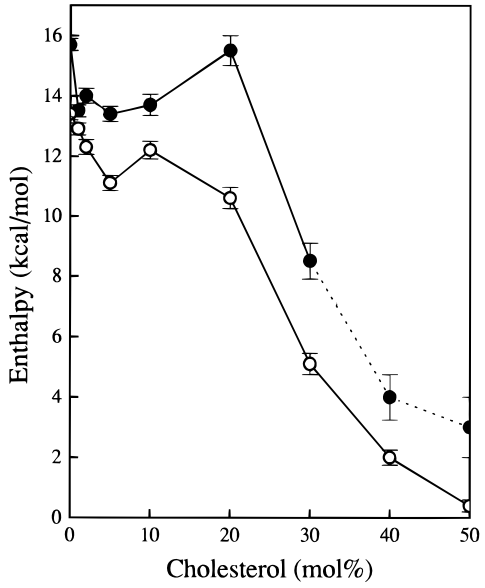

FIGURE 6: Representative plots of the total enthalpy of the L_c/L_α (heating, ●) and the L_α/L_c (cooling ○) phase transitions of aqueous dispersions of the DGDG from elaidic acid-homogeneous *A. laidlawii* membranes as a function of cholesterol concentration.

decreases in their enthalpy and cooperativity are observed with increasing cholesterol levels (Figures 5 and 6), just as observed in the heating experiments. In this range of cholesterol concentrations, the observed cooling exotherms are still attributable to a L_α/L_c phase transitions as shown by FTIR spectroscopy (data not presented). In the higher range of cholesterol contents, the cooling exotherms also broaden significantly, and their midpoint temperatures and enthalpy values decrease sharply. In these cases, however, these data can be attributed, in part, to changes in the nature of the observed exothermic events as cholesterol content increases. This is because high cholesterol contents progressively decrease the rates at which the L_c phase of DGDG forms from the L_β phase when samples are cooled to

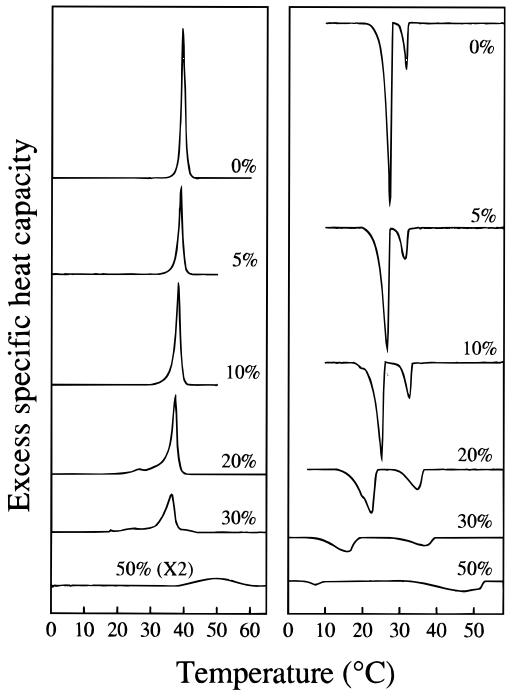

FIGURE 7: Representative DSC heating (left panel) and cooling (right panel) scans of aqueous dispersions of the MGDG from elaidic acid-homogeneous *A. laidlawii* membranes containing various concentrations of cholesterol.

temperatures below 7 °C. As a result, a L_α/L_β phase transition becomes detectable and the L_α/L_β and L_β/L_c phase transitions tend to become separated in temperature as cholesterol content increases. Thus, the weak exothermic event exhibited by samples containing 50 mol % cholesterol is primarily a transition between the L_α and L_β phases. However, even at these elevated cholesterol contents, the L_β phase of DGDG is still metastable with respect to the L_c phase of the lipid, and readily converts to the latter when incubated at low temperatures. Thus, it is clear that when cooled to temperatures below 7 °C, DGDG is very prone to form the L_c phase even when mixed with substantial quantities of cholesterol. This result suggests that cholesteol is poorly miscible with, and tends to be excluded from, DGDG bilayers under all conditions where the hydrocarbon chains are frozen. Our results also suggest that cholesterol is not completely miscible even with the liquid-crystalline phase of DGDG, since at lower cholesterol concentrations regions of pure DGDG must exist which can nucleate L_c phase formation on cooling.

Representative high-sensitivity DSC heating and cooling scans of MGDG from elaidic acid-homogeneous *A. laidlawii* membranes, alone or in the presence of increasing quantities of cholesterol, are shown in Figure 7. As observed also for the DGDG, the MGDG alone forms an L_c phase when exposed to low temperatures, and upon heating the L_c phase converts directly to the H_{II} phase near 39 °C (confirmed by unpublished X-ray and FTIR data from this laboratory). At cholesterol concentrations up to 30 mol %, the relatively sharp, highly enthalpic L_c/H_{II} phase transition endotherm is little affected by the presence of cholesterol, except for a small decrease in transition temperature (Figure 8). At cholesterol concentrations exceeding 30 mol % a broad, second endothermic process is detectable but the overall transition enthalpy does not change significantly (Figure 9). However, at 50 mol % cholesterol, the L_c chain-melting

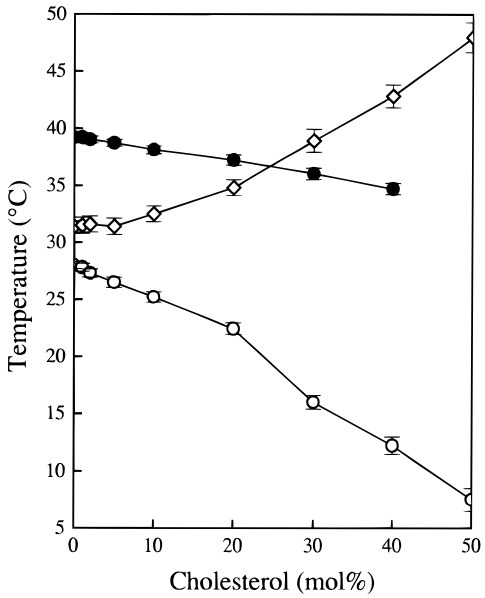

FIGURE 8: Representative plots of the temperatures of the L_c/H_{II} (heating, ●) and of the H_{II}/L_α (cooling, ◇) and L_α/L_β (cooling, ○) phase transitions of aqueous dispersions of the MGDG from elaidic acid-homogeneous *A. laidlawii* membranes as a function of cholesterol concentration. Transition temperatures were corrected for differences in heating and cooling scan rates between the various samples analyzed.

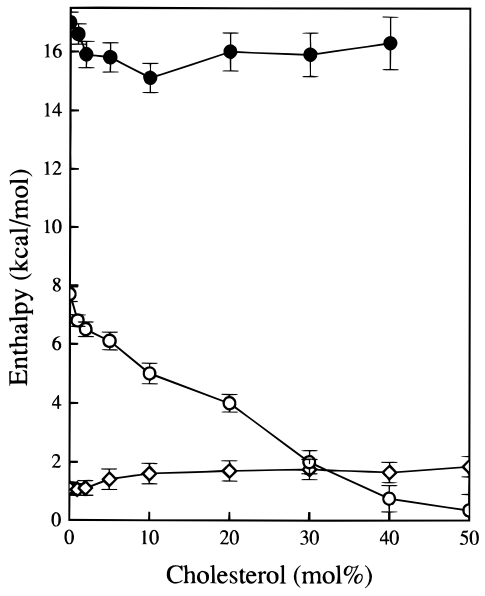

FIGURE 9: Representative plots of the total enthalpy of the L_c/H_{II} (heating, ●) and of the H_{II}/L_α (cooling, ◇) and L_α/L_β (cooling, ○) phase transitions of aqueous dispersions of the MGDG from elaidic acid-homogeneous *A. laidlawii* membranes as a function of cholesterol concentration.

endotherm is completely abolished and only a new endotherm centered near 50 °C persists. As shown below, the new endotherm appearing near 50 °C is probably attributable to a L_α/H_{II} phase transition. Thus cholesterol at low concentration appears to be only sparingly miscible with MGDG at low temperatures, where the L_c phase predominates, but at much higher concentrations cholesterol can apparently be solubilized in MGDG bilayers and can inhibit both L_β and L_c phase formation.

Upon cooling, the *A. laidlawii* MGDG alone exhibits two exotherms, a higher temperature, lower enthalpy H_{II}/L_α near 31 °C and a lower temperature, higher enthalpy L_α/L_β phase

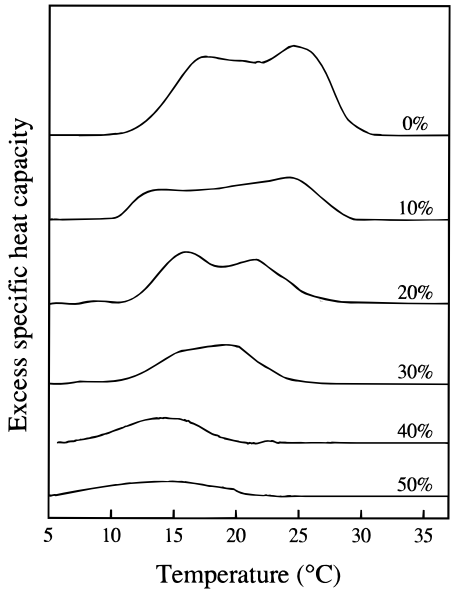

FIGURE 10: Representative DSC heating scans of aqueous dispersions of the total polar lipids from elaidic acid-homogeneous *A. laidlawii* membranes containing various concentrations of cholesterol. Cooling scans were essentially identical and are not illustrated.

transition centered near 28 °C. In this case, however, the presence of cholesterol at all concentrations produces a significant upward shift in the H_{II}/L_α, a small downward shift in the L_α/L_β phase transition temperatures (Figure 8), and a small decrease in the cooperativity of both transitions. At higher cholesterol concentrations, the H_{II}/L_α phase transition becomes more strongly shifted to higher temperatures (Figure 8) and becomes broader but actually *increases* in enthalpy (Figure 9). In contrast, the L_α/L_β phase transition exotherm is shifted strongly to lower temperatures (Figure 8) and is markedly reduced in enthalpy (Figure 9). These results suggest that cholesterol has a much greater miscibility with the L_β and H_{II} states than with the L_c state of elaidic acid-enriched MGDG aqueous dispersions. Moreover, cholesterol incorporation into the MGDG L_α phase bilayer inhibits the formation of the L_c and L_β phases which would normally form at lower temperatures. Thus, the cholesterol which is incorporated stabilizes the L_α phase of aqueous dispersions of MGDG relative to both the L_β and H_{II} phases, thus markedly increasing the temperature range over which the L_α phase is stable upon both heating and cooling.

Representative high-sensitivity DSC heating and cooling scans of the total membrane lipids from elaidic acid-homogeneous *A. laidlawii* cells, in the presence or absence of various amounts of cholesterol, are presented in Figure 10. In the absence of added cholesterol, the total membrane lipids exhibit a fairly broad, ramplike gel to liquid-crystalline phase transition with an overall midpoint temperature near 22 °C, but consisting of at least two major components with transition temperatures near 19 °C and 24 °C. Note that the width of the chain-melting phase transition of the total membrane lipids is much greater than that of the individual major membrane lipids, presumably due only to the membrane lipid polar headgroup compositional heterogeneity, since there is no heterogeneity in the fatty acid composition of these membrane lipids. Note also that the phase transition midpoint temperature of the total membrane lipids is roughly intermediate between the L_β/L_α phase transition temperatures

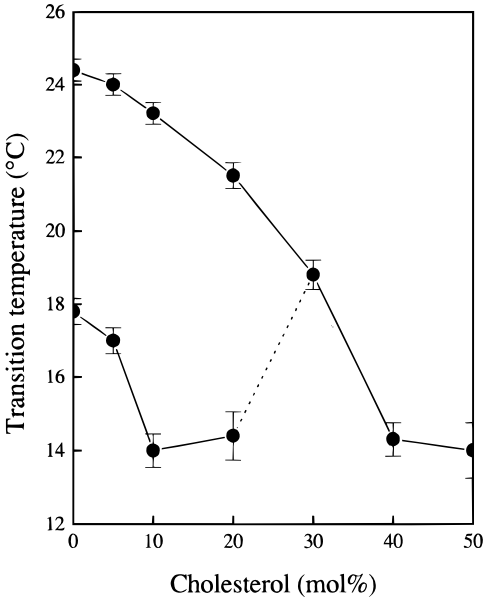

FIGURE 11: Representative plot of the overall chain-melting phase transition midpoint temperature of aqueous dispersions of the total polar lipids from elaidic acid-homogeneous *A. laidlawii* membranes as a function of cholesterol concentration.

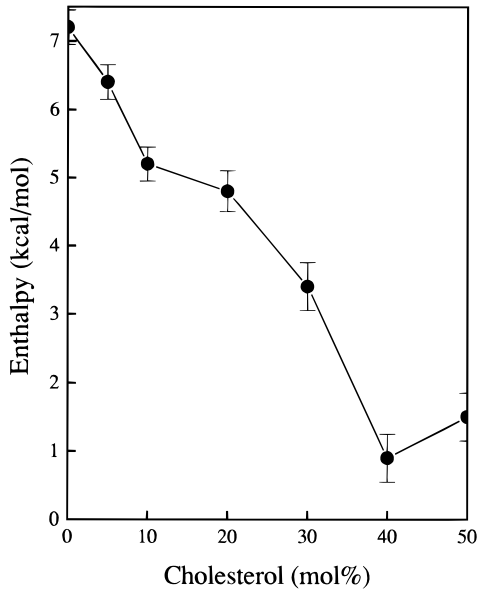

FIGURE 12: Representative plot of the total enthalpy of the chain-melting phase transition of aqueous dispersions of the total polar lipids from elaidic acid-homogeneous *A. laidlawii* membranes as a function of cholesterol concentration.

of the lower-melting PG (11 °C) and DGDG (13.4 °C), and of the higher-melting MGDG (27.9 °C), components. Taken together, these observations indicate that the individual lipid components of the *A. laidlawii* membrane have an appreciable mutual miscibility but that mixing is not ideal.

As the exogenous cholesterol concentration is increased from 0 to 20 mol %, the overall phase transition midpoint temperature of the total membrane lipids is shifted downward (Figure 11) but the two components of this transition remain and the enthalpy of the phase transition is reduced (Figure 12). From 30 to 50 mol % cholesterol, the phase transition continues to decrease in temperature and enthalpy but the transition becomes single component. Moreover, some residual enthalpy remains at 50 mol % cholesterol. Interestingly, the width of the already broad gel to liquid-crystalline phase transition of the total membrane lipids does not appear to increase appreciably as the concentration of cholesterol added increases, at least up to 30 mol % cholesterol. This result is quite different than is observed for the PG component of elaidic acid-homogeneous *A. laidlawii* membranes or for synthetic PC's [see McMullen *et al*. (1993, 1996a)], where a distinct broadening of the chain-melting phase transition is noted even at quite low cholesterol concentrations and where the broadening increases smoothly and progressively with increases in cholesterol incorporation, the phase transition being abolished at 50 mol % cholesterol. However, the *A. laidlawii* neutral glycolipids also exhibit only small increases in the width of their gel to liquid/crystalline phase transitions over the cholesterol concentration range 0−30 mol % and a residual enthalpy at 50 mol % cholesterol, as do the total membrane lipids. It is also possible that cholesterol could increase the mutual solubility of the *A. laidlawii* membrane lipids, particularly at high concentrations, as has been demonstrated in some but not all laterally phase separated ternary cholesterol/phospholipid or glycolipid systems (Silvius, 1992). Such an effect may counteract the intrinsic broadening of the chain-melting phase transition of the individual membrane lipids by cholesterol which is observed especially at high cholesterol levels.

DISCUSSION

We have shown previously that the relative miscibility of cholesterol (McMullen *et al*., 1993, 1996a,b) and various side chain-truncated cholesterol analogues (McMullen *et al*., 1995; Vilchèze *et al*., 1996) with the gel and liquid-crystalline phases of PC and PE bilayers can be determined by the effect of the incorporation of various concentrations of these sterols on the enthalpy of the gel to liquid-crystalline phase transition as monitored by DSC. In binary cholesterol/phospholipid systems exhibiting complete miscibility in both the solid and fluid states, the transition enthalpy decreases smoothly to zero as the sterol content of the mixtures increases from 0 to 50 mol %, in both the heating and cooling modes. Similarly, in binary systems exhibiting only gel-state sterol immiscibility, the enthalpy of the gel to liquid-crystalline phase transition measured in the heating mode first declines (and may reach a plateau) as cholesterol content increases but that in all cases the chain-melting phase transition is not abolished at 50 mol % cholesterol; however, when measured in the cooling mode, the transition enthalpy declines smoothly to zero at 50 mol % cholesterol. Finally, if cholesterol shows limited solubility in both the gel and liquid-crystalline phases of a particular lipid, then a phase transition will remain detectable at 50 mol % sterol in both the heating and cooling modes, with the magnitude of this residual enthalpy providing an approximate measure of the degree of sterol immiscibility in each phase.

It is clear from our DSC results that the miscibility of cholesterol in the gel and liquid-crystalline lamellar phases of the three major lipids of the *A. laidlawii* membrane differs markedly. Thus elaidic acid-enriched PG/cholesterol mixtures exhibit complete miscibility in both the gel and liquid-crystalline phases, while the DGDG/cholesterol and MGDG/cholesterol mixtures exhibit significant immiscibility, particularly in the gel state and particularly at lower cholesterol concentrations. The reason for the relative immiscibility of cholesterol in the neutral glycolipid bilayers generally is probably due to the relatively tightly packed, extensively

hydrogen-bonding bilayers formed by these glycolipids, particularly in the gel state, which tends to exclude cholesterol into separate, laterally segregated domains. The reason for the increased miscibility of cholesterol with MGDG and DGDG bilayers at higher cholesterol concentrations or at higher temperatures may reflect a disruption of glycolipid−glycolipid hydrogen bonding and other interactions, thus loosening the bilayer and permitting a more facile penetration of cholesterol into the glycolipid matrix. In this regard it is interesting to note that we have shown that cholesterol is fully miscible in the gel and liquid-crystalline states of synthetic saturated, mixed-chain and *trans*-unsaturated PC bilayers (McMullen *et al*., 1993, 1995; Vilchèze, 1996), while in saturated (McMullen *et al*., 1996b) or *trans*-unsaturated PE bilayers (McMullen *et al*., 1996a) cholesterol exhibits gel phase immiscibility. Again, calorimetric and spectroscopic data clearly indicate that PE forms higher melting, more densely packed bilayers, particularly in the gel state, as compared to PC, presumably because of the stronger electrostatic and hydrogen-bonding interactions in the polar headgroup region of the former phospholipid (Lewis *et al*., 1993, and references therein). Thus the *A. laidlawii* PG behaves like synthetic and natural PC's in its interaction with cholesterol while the MGDG and DGDG behave more like PE, except that these neutral glycolipids exhibit some immiscibility with cholesterol also in the liquid-crystalline state, whereas PE does not (McMullen *et al*., 1996a). The relative immiscibility of cholesterol on the total membrane lipids of elaidic acid-homogeneous *A. laidlawii* cells is doubtlessly due to the presence of substantial quantities of neutral glycolipids in the cell membrane of this organism (see Table 1).

The difference in the nature of the interaction of cholesterol with PG, and with MGDG and DGDG, is also manifested in the direction and magnitude in the shift in the gel to liquid-crystalline phase transition temperature induced by presence of this sterol. In the case of PG, the L_β/L_α phase transition temperature is increased slightly by the addition of cholesterol, but the upward shift is modest, being only about 3 °C as one approaches 50 mol % cholesterol. However, for the MGDG and DGDG, a substantial decrease in the L_β/L_α phase transition temperatures is observed. In particular this reduction in the phase transition temperature is about 16 and 6 °C, respectively, at 50 mol % cholesterol. Interestingly, a similar slight upward shift in the L_β/L_α phase transition is noted upon the addition of cholesterol to synthetic PCs (McMullen *et al*., 1993, 1995, 1996a) and a similar major downward shift in the L_β/L_α phase transition temperature is noted in synthetic PEs (McMullen *et al*., 1996b). Thus, it appears that in lipids forming low-melting gel state bilayers characterized by relatively weak lipid polar headgroup electrostatic and hydrogen-bonding interactions, cholesterol stabilizes the gel state relative to the liquid-crystalline state, probably by relieving the crowding of the relatively large polar headgroups (in the case of PC), by decreasing the electrostatic repulsion of like charges (in the case of PG), and by providing additional attractive hydrogen-bonding interactions at the phospholipid bilayer polar/apolar interface (both phospholipids) [see McMullen and McElhaney (1996)]. In contrast, the effect of cholesterol incorporation on the higher-melting lipids is to decrease the stability of the gel or crystalline relative to the liquid-crystalline state, presumably by increasing the spacing of the lipid polar headgroups and thus disrupting their initially relatively strong electrostatic and/or hydrogen bonding attractive interactions. In this regard, note that the reduction in the phase transition temperature of the higher melting MGDG component is considerably greater than that of the lower melting DGDG component of identical fatty acid composition, as predicted from their relative phase transition temperatures.

Efrati *et al*. (1986) have proposed that the difference in the exogenous cholesterol incorporation capacities of *Acholeplasma* and *Mycoplasma* species may be due to the much higher membrane neutral glycolipid contents of the former and to a reduced capacity of glycolipids to solubilize cholesterol. This proposal was supported by a statistical analysis of a large number of *Acholeplasma* species and strains which revealed a significant negative correlation between the glycolipid content of the cell membrane and the level of incorporation of cholesterol by growing cells. Also given as evidence for this hypothesis was the observation that maximum cholesterol uptake increases in aging cells in conjunction with decreased neutral glycolipid levels. Moreover, Efrati *et al*. (1981) had shown earlier that the degradation of 90−94% of the PG and DPG from the *A. laidlawii* membrane resulted in a disproportionate decrease in cholesterol uptake. Specifically, although these two phospholipids made up only 30 wt % of the total membrane lipids, their hydrolysis by phospholipase A_2 (and the prior removal of the hydrolysis products from the membrane) resulted in a decrease in cholesterol incorporation of 55%, suggesting that the neutral glycolipids remaining in the membranes must have a reduced capacity for cholesterol uptake. The results of our present DSC study of the interactions of cholesterol with the three major lipids of the *A. laidlawii* membrane would seem to support the hypothesis of Efrati *et al*. (1986) in that the maximum amount of cholesterol which can be incorporated into PG bilayers seems greater than that which can be incorporated into DGDG and MGDG bilayers. In this regard it is noteworthy that the glycosphingolipids from eucaryotic cells generally show a reduced ability to solubilize cholesterol as compared to most phospholipids, and that the ability of glycosphingolipids to solubilize cholesterol also depends more markedly on the exogenous cholesterol concentration than is the case for most phospholipids (McCabe & Green, 1977). However, Efrati *et al*. (1981) stated, without presenting data, that the neutral glycolipid fraction from *A. laidlawii* membrane can solubilize the same maximum amount of cholesterol as can the phospholipid or phosphoglycolipid fractions when cosonicated, a surprising observation in view of the present results and those of others discussed above.

The existence of two pools of cholesterol in the *A. laidlawii* membrane has also been suggested to support the hypothesis of Efrati *et al*. (1986). Thus Davis *et al*. (1984) reported that in intact cells, and in isolated membranes, about half of the cholesterol associated with the membrane exchanges relatively rapidly with egg PC/cholesterol vesicles while the other half exchanges much more slowly. They suggested that the rapidly exchanging cholesterol pool could result from cholesterol binding weakly to the membrane glycolipids and the slowly exchanging pool to cholesterol bound strongly to the membrane phospholipids. However, the removal of most of the phospholipid by phospholipase A_2 hydrolysis resulted in a single kinetic pool of cholesterol which exchanged at a rate only slightly faster than the rate

of the slowly exchanging cholesterol pool in untreated cells, which is not the result predicted if the rapidly exchanging pool in untreated cells is due to glycolipid-bound cholesterol. In this regard, we have indeed confirmed the existence of two structurally different pools of cholesterol in *A. laidlawii* membranes (Monck *et al*., 1993), one solubilized in the lipid bilayer and another in a solid-like form whose location in the membrane could not be determined, and we have also shown that heating or lyophilization and rehydration at high temperature converts the solid-like form of cholesterol into bilayer-solubilized cholesterol. In principle, this conversion of the solid-like cholesterol pool into a lipid bilayer-dispersed form could be due to an increased solubilization of free cholesterol by the membrane neutral glycolipids induced by these treatments. However, whether or not the existence of the solid-like cholesterol pool is related to the presence of the neutral glycolipids in the membrane of this organism remains to be determined.

It is instructive to compare the effects of cholesterol on the L_α/H_{II} phase transition of synthetic DEPE and the MGDG from elaidic acid-homogeneous *A. laidlawii* membranes. The L_α/H_{II} phase transition temperature of DEPE has been shown to decrease moderately from 0 to 10 mol % cholesterol, to plateau at 10 to 30 mol % cholesterol, and then to increase markedly at higher cholesterol concentrations, while the phase transition enthalpy increases markedly (over 3-fold) and apparently linearly over this same sterol concentration range. Moreover, the DEPE L_α/H_{II} phase transition DSC endotherm is clearly two-component over this entire range of cholesterol concentrations (Epand & Bottega, 1987; Cheetham *et al*. 1989). In contrast, the L_α/H_{II} phase transition temperature of MGDG increases regularly and supralinearly over the cholesterol concentration range 0−50 mol % and the transition enthalpy increases by less than 2-fold from 0 to 20 mol %, where the enthalpy values plateau. Moreover, the MGDG DSC exotherms are not obviously two-component except at the highest cholesterol concentrations. Thus, just as the effect of cholesterol on the L_α/H_{II} phase transition of synthetic and natural PEs depends to a considerable extent on the length and degree of unsaturation of the fatty acyl chains (Dekker *et al*., 1983; Epand & Bottega, 1987; Cheetham *et al*., 1989), so too does it depend on the polar headgroup structure of the lipid when the composition of the fatty acyl chains are held constant.

The effect of any additive on the L_α/H_{II} phase transition temperature of a nonlamellar phase-forming lipid is usually explained on the basis of the effective molecular shape of that additive and by its effect on the dynamic shape of the host lipid molecules with which it interacts [see Lewis *et al*. (1996)]. Because of the small size and cross-sectional area of its polar headgroup in comparison to the relatively large size and cross-sectional area of its nonpolar fused ring system, the cholesterol molecule is usually considered to have an inverted cone shape and should therefore promote H_{II} phase formation. However, cholesterol also orders the hydrocarbon chains of the host lipid bilayer and increases its hydrophobic thickness, factors which should reduce the propensity of the host lipid to form a reversed phase, since this phase is characterized by more highly disordered hydrocarbon chains and by monolayers of decreased thickness. Moreover, the incorporation of cholesterol will increase the spacing of the polar headgroups in the bilayer, which for the small, strongly interacting polar headgroups of PE and MGDG molecules, should decrease the intrinsic radius of curvature of each monolayer and thus the propensity to form the H_{II} phase. Similarly, the reduction of the L_β/L_α phase transition temperature noted with both synthetic and natural PEs and MGDG can be explained by the effect of cholesterol in disordering the hydrocarbon chains of the host lipid bilayer at low temperatures, which also thus decreases the hydrophobic thickness of the lipid bilayer and reduces the magnitude of the attractive interactions between the polar headgroups of adjacent phospho- or glycolipid molecules, thus promoting L_α over L_β phase formation. However, additional work will be required to explain why the balance of forces always favors H_{II} phase formation in elaidic acid-enriched MGDG but may favor either L_α or H_{II} phase formation in DEPE, depending on the concentration of cholesterol.

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