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Chain Length Dependent Modification of Lipid Organization by Low Levels of 25-Hydroxycholesterol and 25-Hydroxycholecalciferol. A Laser Raman Study†

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ABSTRACT: We have used Raman spectroscopy to investigate the thermal transitions of multibilayered liposomes composed of lecithins, i.e., dilauroyllecithin, dimyristoyllecithin, dipalmitoyllecithin, distearoyllecithin, or egg lecithin, plus 5-cholesten-3,25-diol (25-hydroxycholesterol), 25-hydroxycholecalciferol, and vitamin D_3. We recorded the CH-stretching (2800–3000-cm^{-1}) regions of the Raman spectra at various temperatures and employed plots of temperature vs. the intensity of the 2880- or 2930-cm^{-1} bands relative to that of the 2850-cm^{-1} feature, i.e., the ratios I_{2880}/I_{2850} and I_{2930}/I_{2850}, to estimate thermal transitions. These plots show multiple discontinuities, each of which may be ascribed to a state change of a separate phase with distinctive proportions of lecithins and cholesterol derivatives. Low concentrations of 25-hydroxycholesterol and 25-OH-D_3 (≥0.2 mol %) abolish the pretransition and split the main transitions of dilauroyllecithin (4 °C) and dimyristoyllecithin (23 °C) into two. The midpoint of the new small transition centers at about 3–4 °C lower than those of the respective main transitions of dilauroyllecithin and dimyristoyllecithin. A further increase in the molar ratio of 25-hydroxycholesterol and 25-hydroxycholecalciferol decreases the amplitudes of the new and the main transitions (dilauroyl- and dimyristoyllecithin). The transitions of dipalmitoyllecithin and distearoyllecithin at 2 mol % concentrations of either sterol remain unaffected. There was no splitting in the main transition of either dipalmitoyllecithin or distearoyllecithin in the presence of these sterols. The perturbing effect of the 25-hydroxysterols follows the order dilauroyllecithin > dimyristoyllecithin > dipalmitoyllecithin > distearoyllecithin. Our data suggest that 25-hydroxysterols disorganize phospholipids of 12–14-carbon acyl-chain length more than those with 16–18-carbon chains. As assessed by changes in the intensities of the 1653-cm^{-1} C=C stretching band, interaction with phospholipids also involves the aliphatic double bonds of 25-hydroxycholecalciferol.

Most cells can derive cholesterol from two sources, namely de novo biosynthesis from acetyl coenzyme A (Sabine, 1977) or receptor-mediated uptake and liposomal degradation of plasma low-density lipoprotein (Brown & Goldstein, 1979). The control mechanisms for cellular cholesterol homeostasis are not perfectly known. Endogenous cholesterol biosynthesis is regulated by a feedback mechanism that controls the activity of 3-hydroxy-3-methylglutaryl-CoA reductase (HMG-CoA reductase), the rate-limiting enzyme in cholesterogenesis, located in reticulum endoplasmic membranes (Rodwell et al., 1976). As shown by Kandutsch & Chen (1975), certain oxidation products of cholesterol, rather than cholesterol itself, regulate sterol biosynthesis at the HMG-CoA reductase level. The list of the oxygenated sterols that have this capacity continues to increase (Schroepfer et al., 1977, 1980; Shakespeare & Wilton, 1980) and includes several cholesterol precursors (Gibbons et al., 1980) and some oxygenated derivatives of vitamin D_3 (Philippot et al., 1976).

The several sterically dissimilar sterols, all more polar than cholesterol, suppress HMG-CoA reductase activity suggests the possibility that this endoplasmic reticulum enzyme is regulated by the state of its membrane lipid environment, as is also indicated by Arrhenius plot discontinuities of HMG-CoA reductase (Mitropoulos & Venkatesan, 1977, Sabine & James, 1976; Philippot & Wallach, 1979), signifying cooperative changes of phase within the enzyme or its microenvironment (Raison, 1973).

In order to elucidate the mechanism of suppression of HMG-CoA reductase activity by these oxygenated sterols, we have used Raman spectroscopy to study the interactions be-

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tween 25-OH-Ch[1] or 25-OH-D_3 and model membranes as a function of temperature. The thermal behavior of lecithin liposomes containing 25-OH-Ch or 25-OH-D_3 was monitored in the range of 2800–3000 cm^{-1}, where Raman scattering due to CH-stretching vibrations provides information about lateral interactions between acyl chains of phospholipids (Gaber & Peticolas, 1977). We find that the structure of the CH-stretching region changes in a temperature dependent fashion upon incorporation of small proportions of 25-OH-Ch (2 mol %) or 25-OH-D_3 (0.2 mol %) and that the variation depends on the length of the methylene chain of phospholipids. We also note a change in the C=C stretching region, suggesting an effect on the structure of 25-OH-D_3 caused by the association of this sterol with phospholipid.

Materials and Methods

Synthetic DLL, DML, DPL, and DSL, as well as EYL, were purchased from Sigma (St. Louis, MO). 25-OH-Ch was purchased from Steraloids, Inc. (Milton, NH). 25-OH-D_3 was a gift from Upjohn Co. (Kalamazoo, MI).

Multilamellar liposomes were prepared by dissolving weighed amounts of lipid and 25-OH-Ch/25-OH-D_3 in chloroform, followed by gassing with nitrogen and then drying under vacuum for several hours. The dried samples were then hydrated with water. Prior to spectroscopic measurements, sealed hydrated samples were heated for 3 h in a water bath at temperatures 5 ± 2 °C higher than transition temperatures of the phospholipids and were periodically agitated in a Vortex mixer. Raman spectra were recorded on a Ramalog 4 Raman spectrometer (Spex Industries, Metuchen, NJ). The samples, sealed in Kimax capillaries (1-mm i.d.), were irradiated by an Ar^+ ion laser (Spectra Physics, Model 164) tuned at 488 nm. Sample temperatures were controlled by a flow of temperature-regulated nitrogen gas, by using a Harney–Miller cell. The temperature in the cell was measured continuously with a thermistor placed close to the laser beam (Wallach et al., 1979). Measured temperatures with 2–3 °C lower than sample temperatures, according to calibration curves with pure phospholipids and fatty acids. The transition temperatures reported in this paper are uncorrected.

Conventional scanning Raman spectroscopy was as detailed in Wallach et al. (1979). Specific parameters are given in the figure legends. Multiplex analyses were carried out by utilizing a PAR (Princeton Applied Research) optical multichannel analyzer system (OMA 1) as in Verma & Wallach (1982). We collected 1000 scans (32.8 ms each) per control and data point (i.e., 50 times each). Recording of the differences takes 50 s. One difference data point thus takes 2.5 min. For temperature scans, we obtain one ~250-cm^{-1} interval (as a difference spectrum) per degree (−10 to 50 °C) and scan both up and down.

Results

CH Stretching and Thermal Transition. Representative CH-stretching spectra (2800–3000 cm^{-1} of DPL liposomes, obtained at various temperatures by multiplex analysis) have normal shapes and resolution as compared to the one obtained by conventional recording (Figure 1). We calculate the intensity of the CH-stretching bands using the base line joining the intensity minima of the spectrum. These minima generally lie at 2820 ± 5 and 3000 ± 5 cm^{-1}. Slight differences in

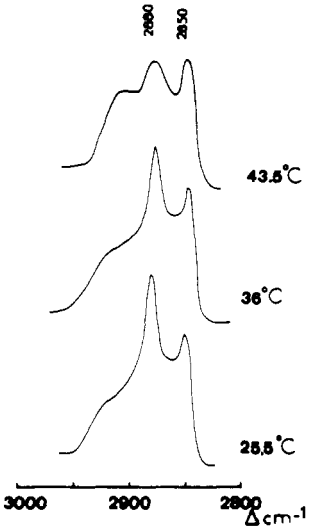

FIGURE 1: OMA Raman spectra in the 2800–3000-cm^{-1} (CH-stretching) region of DPL at 25, 36, and 43 °C. Laser power 500 mW; 1000 scans (32.8 ms each). No delay.

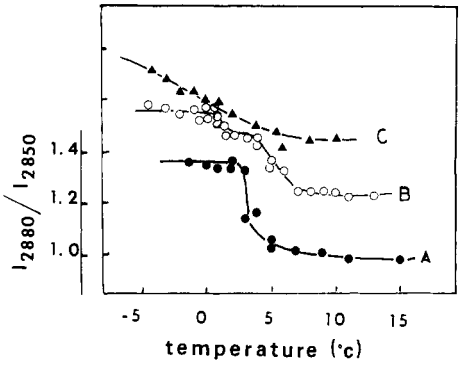

FIGURE 2: Plots of I_{2880}/I_{2850} vs. temperature for DLL ± 25-OH-D_3: (●) DLL; (○) DLL + 25-OH-D_3 (1/0.002, M/M); (▲) DLL ± 25-OH-D_3 (1/0.02, M/M). Error in each data point is less than 3%. For clarity, I_{2880}/I_{2850} is displaced upward by 0.2 for curves B and C.

I_{2880}/I_{2850} occur between OMA and scanning analyses. These arise from differences in its distribution of bands and background in various channels of the SIT. Slight difference in intensity ratios (as noted by these two analyzing technique) does not affect the numerical values of thermal transitions.

A significant difference lies in data acquisition time. In conventional spectroscopy (computer driven), scanning from 2800 to 3050 cm^{-1} takes 300–350 s (for single scan), with employment of 1.5 s as the maximum time per data point, while OMA spectroscopy requires 30 s.

The transition properties of liposomes with various proportions of phospholipid ± 25-OH-Ch and 25-OH-D_3, as reported below, are calculated from plots of I_{2880}/I_{2850} vs. temperature. Individual data points illustrate the small error in the method. Reproducible thermal data were obtained from at least three different sample preparations.

Effects of 25-OH-D_3. (*A*) *DLL.* The main transition of DLL occurs at 3–5 °C (Figure 2A). The effects of various concentrations of 25-OH-D_3 on this transition are given in Figure 2B,C. At 1/0.002 DLL/25-OH-D_3 (M/M), the main transition splits into two (Figure 2B). A 10-fold increase in the proportion of 25-OH-D_3 concentration (1/0.02, M/M) broadens both transitions so that they merge (Figure 2C).

(*B*) *DML.* DML liposomes give a pretransition around 8 °C and a main transition at 23 °C (Figure 3A). At a 1/0.002 (M/M) DML/25-OH-D_3 ratio (Figure 3B), the main tran-

[1] Abbreviations: DLL, dilauroyllecithin; DML, dimyristoyllecithin; DPL, dipalmitoyllecithin; DSL, distearoyllecithin; EYL, egg yolk lecithin; 25-OH-Ch, 25-hydroxycholesterol (5-cholesten-3,25-diol); 25-OH-D_3, 25-hydroxycholecalciferol; OMA, optical multichannel analysis; SIT, silicon-intensified tube.

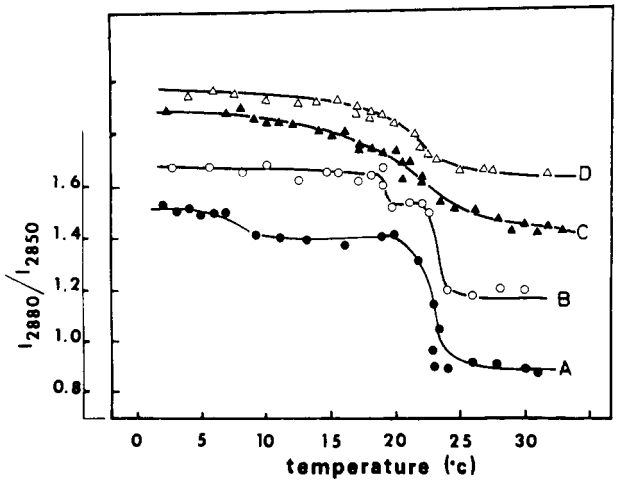

FIGURE 3: Plots of I_{2880}/I_{2850} vs. temperature for DML ± 25-OH-D_3: (●) DML; (○) DML + 25-OH-D_3 (1/0.002, M/M); (▲) DML + 25-OH-D_3 (1/0.02, M/M); (△) DML + 25-OH-D_3 (1/0.2, M/M). Error in each data point is less than 3%. For clarity, I_{2880}/I_{2850} is displaced upward by 0.2 for curves B–D.

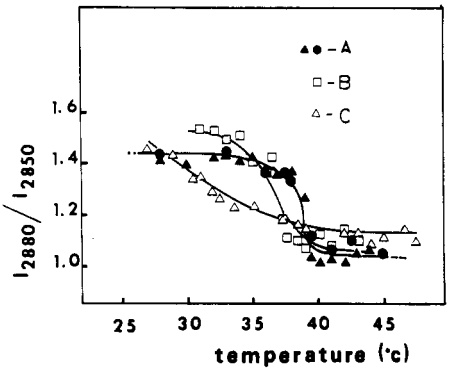

FIGURE 4: Plots of I_{2880}/I_{2850} vs. temperature for DPL ± 25-OH-D_3: (●) DPL; (▲) DPL + 25-OH-D_3 (1/0.02, M/M); (□) DPL + 25-OH-D_3 (1/0.06, M/M); (△) DPL + 25-OH-D_3 (1/0.2, M/M). Error in each data point is less than 3%.

sition shifts almost imperceptibly, a new transition develops and decreases slightly at 19.5 °C, and the pretransition disappears. We do not observe any splitting of the main transition at a DML/25-OH-D_3 ratio of 1/0.02, although substantial reduction and broadening do occur (midpoint 21 °C, Figure 3C). A 10-fold increase in 25-OH-D_3 (1/0.2) further broadens the transition (Figure 3D). In curves 3C and 3D, the inflection point is between 20 and 24 °C.

(*C*) *DPL.* The main transition of DPL near 39 °C (Figure 4A) does not respond drastically to lower proportions of 25-OH-D_3. No effect is observed at a 1/0.02 ratio (M/M), whereas this proportion of 25-OH-D_3 in DLL completely wipes out the main transition and drastically modifies it in DML. A 3-fold increase in the proportion of 25-OH-D_3 to 1/0.06 (M/M) shifts the main DPL transition slightly down to ~37 °C (midpoint), leaving it comparatively broad (Figure 4B). In liposomes containing DPL and 25-OH-D_3 at a ratio of 1/0.2 (M/M), the main DPL transition is broadened, reduced in intensity, and shifted to 30 °C (Figure 4C).

(*D*) *DSL.* The main transition of DSL occurs at 50 °C (Verma et al., 1980). In contrast to its effects of liposomes composed of DLL, DML, and DPL, 25-OH-D_3 does not alter the thermal transition of DSL, even at a 1/0.2 molar ratio. We did not study the effect of 25-OH-D_3 at proportions higher than 20 mol %.

Effect of Vitamin D_3. We studied the effect of vitamin D_3 on the thermal transition of phospholipids at molar ratios of

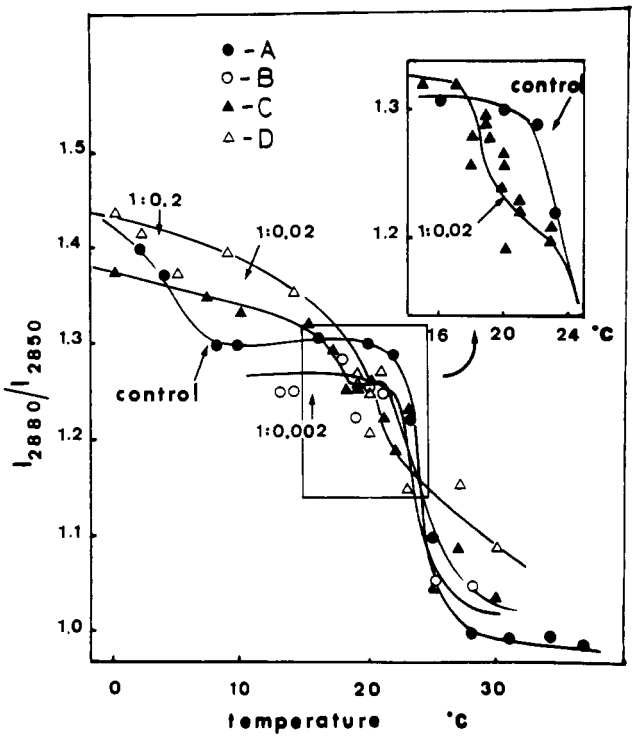

FIGURE 5: Plots of I_{2880}/I_{2850} vs. temperature for DML ± 25-OH-Ch: (●) DML; (○) DML + 25-OH-Ch (1/0.002, M/M); (▲) DML + 25-OH-Ch (1/0.02, M/M); (△) DML + 25-OH-Ch (1/0.2, M/M). Error in each data point is less than 3%. The inset is enlarged on the I_{2880}/I_{2850} scale for curves A and C. The Raman spectra in the CH-stretching region at different temperatures were collected by a scanning technique and, therefore, at different values for the ratio I_{2880}/I_{2850}.

1/0.002 to 1/0.02. Unlike 25-OH-D_3, none of these proportions significantly modify the order ⇌ disorder transition of DML.

Effect of 25-OH-Ch. (*A*) *DML.* At a proportion of 1/0.002 (DML/25-OH-Ch, M/M), 25-OH-Ch does not perturb the main transition of DML (Figure 5A). However, at a molar ratio of 1/0.02, splitting occurs in the main transition (23 °C) (figure 5B) with a small transition detected by scanning Raman spectroscopy near 17 °C (midpoint) (Figure 5, inset), while the main transition broadens without an appreciable shift. At a 1/0.2 proportion (M/M), 25-OH-Ch drastically perturbs the main DML transition (Figure 5C). Both split transitions widen. The flat region from 18 to 20 °C seen in the curve at molar ratio 1/0.02 is reduced to ~5 °C. The low-temperature transition is much broader (midpoint ~15 °C).

(*B*) *DPL.* Figure 6 represents the effect of 25-OH-Ch on the thermal transition of DPL. At a DPL/25-OH-Ch ratio of 1/0.02, the main transition and pretransition of DPL are as in the absence of 25-OH-Ch (Figure 6A). At a molar ratio of 1/0.01, the pretransition remains unchanged, while the main transition is reduced and widened (Figure 6B). An increase in 25-OH-Ch proportion to a ratio of 1/0.2 abolishes the pretransition. Concomitantly, the main DPL transition is considerably reduced and broadened to completion at 48 °C (Figure 6C).

(*C*) *Egg Lecithin.* EYL yields a cooperative order ⇌ disorder transition at −5 °C (Verma et al., 1980). At an EYL/25-OH-Ch ratio of 1/0.07, the EYL transition, indicated by I_{2880}/I_{2850}, splits, with one peak at −8 °C and a smaller one at 0 °C. Two bands at 2885 and 2980 cm^{-1} overlap between 2 and 5 °C. Below −5 °C, there is a single band at 2880 cm^{-1}. The plot of I_{2930}/I_{2850} vs. temperature shows a single transition

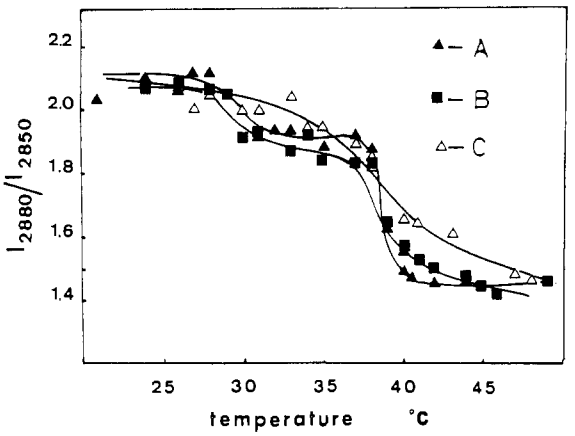

FIGURE 6: Plots of I_{2880}/I_{2850} vs. temperature for DPL + 25-OH-Ch: (▲) DPL + 25-OH-Ch (1/0.02, M/M); (■) DPL + 25-OH-Ch (1/0.1, M/M); (△) DPL + 25-OH-Ch (1/0.2, M/M). For DPL, see Figure 4.

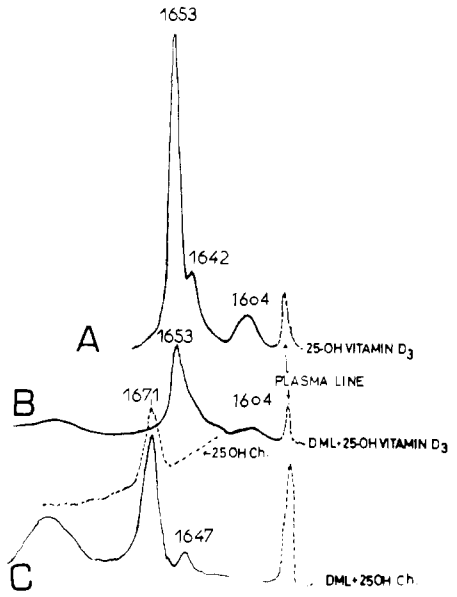

FIGURE 7: Scanning spectra of DML + 25-OH-D_3 (D_2O) in the 1600–1700-cm^{-1} region. Laser power 500 mW; slit resolution 6 cm^{-1}, maximum and minimum time per data point of 1.5 and 1.0 s, respectively. (A) 25-OH-D_3; (B) 25-OH-D_3 + DML; (C) DML + 25-OH-Ch. The Raman spectrum for 25-OH-Ch is marked by dotted lines.

at −7 °C (midpoint). The data suggest that two phases form in the presence of 25-OH-Ch.

DML plus 25-OH-D_3 (1600–1700-cm^{-1} Region). We have used scanning spectroscopy to record the Raman spectra of 25-OH-D_3 (in D_2O) ± DML in the 1600–1700-cm^{-1} region at 21 °C (figure 7). 25-OH-D_3 alone yields two strong features at 1653 and 1642 cm^{-1} and a weak feature at 1604 cm^{-1} in this region (Figure 7A). The strong 1653-cm^{-1} band is tentatively assigned to the double bonds between C5=C6 and C7=C8 (conjugated in the open chain) and the 1642-cm^{-1} feature to C10=C19 double bond. D_2O suspensions of 25-OH-Ch show a strong band at 1671 cm^{-1}, assigned to the ring double bonds. A detailed band analysis and analysis of internal motions of these two derivatives are in progress.

The 1653-cm^{-1} feature of 25-OH-D_3 alters upon incorporation of the sterol into DML liposomes (Figure 7B). The total width of the 1653-cm^{-1} band increases from 8 cm^{-1} at half-height (Figure 7A) to 12 cm^{-1} (Figure 7B). The 1653-cm^{-1} feature in DML ± 25-OH-D_3 is asymmetric, probably because the 1642-cm^{-1} band merges with it. The height of the 1653-cm^{-1} band is also reduced. In pure 25-OH-D_3, the ratio I_{1604}/I_{1653} is 0.13; it increases to 0.24 in the liposomes. The intensity ratio of the 1604-cm^{-1} feature, as compared with the nearby laser line (1590 cm^{-1}), is constant in both 25-OH-D_3 in suspension and Ch–DML liposomes (1604 cm^{-1} can be used as internal reference). The 1653-cm^{-1} band decreases relative to the laser line. The ring double bond vibrations (1671 cm^{-1}) of 25-OH-Ch do not change upon incorporation into DML liposomes.

Discussion

Raman spectroscopic analyses have shown that high molar ratios of cholesterol in lecithin multibilayers broaden the main gel ⇌ liquid-crystal transition temperature (Lippert & Peticolas, 1971). However, plots of I_{2880}/I_{2850} vs. temperature for low molar ratios of 25-OH-Ch and/or 25-OH-D_3 to lecithin reveal multiple discontinuities. In parallel to our previous finding (Verma et al., 1980), we reason that each of these spectroscopic transitions represents a phase with distinct proportions of 25-OH-Ch and/or 25-OH-D_3 and lecithins.

At very low concentrations, 25-OH-Ch and 25-OH-D_3 split the main thermal transition of DLL and DML. The splitting occurs at the lowest studied proportions of 25-OH-D_3 to lecithin (0.002/1, M/M). At a 10-fold higher sterol/lecithin proportion (0.02/1, M/M), splitting is also observed with 25-OH-Ch. No splitting occurs with DPL and DSL where the main thermal transitions remain unaffected up to a 0.02/1 sterol/lecithin ratio but broaden at higher sterol proportions.

The low-temperature transition (Figures 2, 3, and 5) is small and shifts by 2–3 °C to −1.5 °C in DLL and to 19.5 °C in DML in the presence of low levels of 25-OH-D_3 and 25-OH-Ch. The higher temperature transition is reduced in height, becomes broad, and shifts to higher temperatures by 2–3 °C (in DLL, at 5 °C; in DML, at 24 °C). As the concentration of 25-OH-Ch/25-OH-D_3 is further increased (1/0.2 in DML), both these transitions broaden considerably, decrease in height, and seem to merge (Figures 2, 3, and 5).

Both 25-OH-D_3 and 25-OH-Ch perturb the thermal transitions of DLL and DML at low concentrations, but the transitions of DPL and DSL are affected at only higher concentrations of these agents. The data suggest that the shorter the chain length of the phospholipid the less the amount of 25-OH-D_3 or 25-OH-Ch is needed to alter their organization. The effect of 25-OH-Ch and 25-OH-D_3 is in order of DLL > DML > DPL > DSL. The effect need not follow this order in the case of unsaturated phospholipids, but to date, we have only tested EYL, which has one double bond in one of the acyl chains and a mixed acyl chain length. A systematic study of this question is in progress.

The thermal data indicate that 25-OH-Ch and 25-OH-D_3 do not mix in proportions >2 mol % with phospholipids of 12–14-C chain length. However, these derivatives engender a transition-broadening effect similar to that of cholesterol in lecithins of chain lengths of more than 16 carbon atoms but at lower proportions than cholesterol. This may derive from the extra OH group at almost opposite ends of these molecules. Raman (Bush et al., 1980) and infrared (Umenmura et al., 1980) studies of the phospholipid–cholesterol complex indicate that the OH group of cholesterol lies close to the carbonyl groups of phosphatidylcholines. It is difficult to predict whether one or both hydroxyls of 25-OH-Ch and 25-OH-D_3 will align with the head group of phosphatides. However, space-filling models suggest that 25-OH-D_3 but not 25-OH-Ch can be folded so that both hydroxyls align close to phosphatide carbonyls. The changes in the 1653-cm^{-1} feature may be compatible with this. The arrangement just described will

hinder phosphatide interactions between phosphatide molecules, leading to phase separation and ultimately liposome destabilization. Alternatively, one of the two OH groups of 25-OH-Ch and 25-OH-D_3 may prefer an apolar environment, making molecular folding unnecessary. In this case, the behavior of hydroxyl derivatives would be expected to approach that of cholesterol. Our data do not support this hypothesis, at least for DLL and DML.

It is interesting to consider the proportion of 25-OH-Ch and 25-OH-D_3 that will mix with phospholipids and relative to the concentrations than inhibit cholesterol biosynthesis in living cells such as normal and leukemic (L_2C) lymphocytes. The lipid content (phospholipid plus cholesterol; Philippot et al., 1977) and the cell concentration in typical experiments on inhibition of cholesterol synthesis (5×10^6 cells/mL) indicate that the concentrations of 25-OH-Ch and 25-OH-D_3 required for half-inhibition (Philippot et al., 1976) represent 0.4–1.4% of normal cell lipids of 0.3–0.4% of L_2C cell lipids. These values are very close to those that produce transition splitting in DLL and DML.

Acknowledgments

We express our gratitude to Dr. John C. Babcock (Upjohn Co., Kalamazoo, MI) for his gifts of 25-hydroxyvitamin D_3 and vitamin D_3.

Registry No. DLL, 18285-71-7; DML, 13699-48-4; DPL, 2644-64-6; DSL, 4539-70-2; 25-OH-Ch, 2140-46-7; 25-OH-D_3, 19356-17-3; vitamin D_3, 67-97-0.

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