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Thermal Behavior of Synthetic Sphingomyelin–Cholesterol Dispersions†

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ABSTRACT: The thermotropic behavior of aqueous dispersions of palmitoylsphingomyelin–cholesterol and lignocerylsphingomyelin–cholesterol mixtures has been examined by high-sensitivity differential scanning calorimetry. When less than 25 mol % cholesterol is mixed with either sphingomyelin, the calorimetric endotherm is composed of a sharp and a broad component. The sharp-component enthalpy change decreases as the mole percent cholesterol increases with the extrapolated zero enthalpy point being 25 to 30 mol %. With palmitoylsphingomyelin, the temperature of maximum heat capacity of the sharp component decreases monotonically with increasing cholesterol content, while the lignocerylsphingomyelin sharp-component maximum remains constant until more than 20 mol % sterol is present. The broad-component enthalpy change maximizes at 3–4 kcal/mol between 10 and 20 mol % cholesterol and decreases as the ratio of cholesterol is increased or decreased from this range for both sphingomyelins. The results are compared with those from a previous study on dipalmitoylphosphatidylcholine–cholesterol mixtures and are interpreted as evidence for the coexistence of cholesterol-rich and cholesterol-poor phases.

Sphingomyelin is a primary constituent of many mammalian membranes, comprising up to 60 mol % of the total phospholipid in some tissues (Rouser & Solomon, 1969; Broekhuyse, 1969). The relative concentration of this lipid is a parameter of some physical significance since membrane permeability and osmotic fragility are highly correlated with sphingomyelin content (Hertz & Barenholz, 1975; Kirk, 1977; Borochov et al., 1977). This lipid also appears to be the binding site for acetylcholinesterase (Watkins et al., 1977) and for certain cytolytic toxins (Linder et al., 1977). In addition, sphingomyelin is intimately involved with the aging process and a number of pathological conditions. For example, the sphingomyelin content of human aorta and lens tissue increases several fold over a normal lifespan (Rouser & Solomon, 1969; Broekhuyse, 1969), while proper fetal lung development is characterized by a relative decrease in this lipid during the last few weeks of gestation (Gluck et al., 1971). Portman (1970) has associated an elevation of sphingomyelin concentration with the initiation of atherosclerosis in squirrel monkeys, and Hughes (1972) has reported that the amount of this lipid is elevated in dystrophic muscle tissue. For these reasons, the study of the interaction of sphingomyelin with other lipids is of clinical significance as well as being of interest to researchers concerned with fundamental questions of membrane structure.

Of particular interest is the characterization of sphingomyelin–cholesterol mixtures. Cholesterol is often found in significant quantities in membranes which contain a relatively high proportion of sphingomyelin (Patton, 1970), and it has been suggested that these two lipids form a complex (Patton, 1970; Vandenheuvel, 1963). Recently, Demel et al. (1977)

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Table I: Fatty Acid Composition of Natural Sphingomyelins

source	fatty acid[a] 16:0	18:0	24:0	24:1	ref
bovine brain	2.4	36.1	2.7	35.0	*b*
bovine brain	3.9	44.8	5.1	27.4	*c*
bovine brain	2.8	35.9	9.7	30.8	*d*
ovine brain		65.5	10.9	5.9	*d*
porcine brain	4.5	30.1	13.2	21.3	*e*
human brain					*f*
grey matter	8.7	76.8	1.3	4.4	
white matter	9.3	43.0	5.3	22.8	
myelin	6.3	45.2	6.5	28.6	
bovine lens	32.9	5.1	3.8	30.9	*g*
rabbit lens	44.1	10.4	3.7	11.7	*g*
human lens	63.7	1.7	5.3	13.9	*h*
human cataract	62.3	5.1	4.4	7.3	*h*
bovine spinal cord	3.4	54.5	3.7	14.7	*i*
bovine erythrocyte ghost	22.5	5.2	39.5	6.5	*j*
human erythrocyte ghost	30.0	7.8	15.7	30.5	*k*

[a] Fatty acids specified as *m*:*n* where *m* is the number of carbon atoms and *n* is the number of double bonds. Table values are percent of total. [b] Untracht & Shipley, 1977. [c] Shipley et al., 1974. [d] Barenholz et al., 1976. [e] Pullarkat & Reha, 1976. [f] O'Brien & Sampson, 1965. Data from this reference have been averaged over age groups. [g] Anderson et al., 1969. [h] Obara et al., 1976. [i] Hertz & Barenholz, 1975. [j] Demel et al., 1977. [k] Van Dijck et al., 1976.

have presented the results of calorimetric experiments which imply that there may be a preferential affinity of cholesterol for beef brain sphingomyelin in ternary mixtures with phosphatidylcholines or phosphatidylethanolamines in which phase separation of the phospholipids occurs. The primary observation leading to this assertion is that the inclusion of cholesterol in such mixtures results in a selective decrease in the enthalpy change of the gel to liquid-crystalline phase transition of the sphingomyelin phase as opposed to that arising from the other phospholipid. When cholesterol is added to beef brain sphingomyelin alone, a diminution in the gel to liquid-crystalline transition enthalpy change can be observed as the cholesterol content is increased. The precise nature of the sphingomyelin–cholesterol interaction remains obscure, however, because beef brain sphingomyelin has a heterogeneous fatty acid composition (Table I). Previous work from this laboratory (Barenholz et al., 1976), as well as that of Shipley et al. (1974), has demonstrated that this lipid exhibits a complex melting behavior in its own right.

In order to more fully characterize sterol–phospholipid interactions, we have examined aqueous dispersions of cholesterol with synthetic sphingomyelins of homogeneous fatty acid composition, using high sensitivity scanning calorimetry. The sphingomyelins utilized in these studies contained saturated, linear acyl chains with 16 (palmitoyl), 18 (stearoyl), or 24 (lignoceryl) carbon atoms. As can be seen by reference to Table I, these species, along with the monounsaturated nervonic acid, comprise the bulk of the fatty acid residues found in sphingomyelins isolated from natural sources. In this publication, we report the results of experiments performed with palmitoyl- and lignocerylsphingomyelin. Due to the unusual properties of systems containing stearoylsphingomyelin, the results of studies with this lipid will be reported separately.

Experimental Procedures

Materials. DL-*erythro*-*N*-Palmitoylsphingosinephosphorylcholine and DL-*erythro*-*N*-lignocerylsphingosinephosphorylcholine were generously supplied by Professor D. Shapiro of the Weizmann Institute of Science, Rehovot, Israel. Previously reported analyses have shown that these lipids contain at least 99.8% of the desired fatty acid and less than 5% of the dihydrosphingosine base (Barenholz et al., 1976). Lipid quality was further enhanced by silicic acid chromatography and acetone precipitation. The resulting sphingomyelins appear to be highly pure when analyzed by thin-layer chromatography on silica gel plates developed in 65:25:3.5:1.5 (v/v) chloroform–methanol–ammonia–water with a loading of approximately 1 μmol of lipid. Cholesterol was purchased and further purified, and aqueous solutions were formulated as described elsewhere (Estep et al., 1978).

Methods. Lipid dispersions were prepared in 50 mM KCl as described previously (Estep et al., 1978) with the following exceptions: Sphingomyelin–cholesterol mixed films used in the preparation of aqueous dispersions were prepared by the evaporation of solutions made of 7:1 (v/v) chloroform–methanol rather than pure chloroform to prevent selective precipitation of either lipid component; the dry lipid films were warmed to 50–60 °C before the addition of aqueous medium and subsequent incubation was continued within this temperature range. The calorimetric procedures and data analysis were also described previously (Estep et al., 1978). Samples were quantitatively recovered from the calorimeter cells by removal with a syringe followed by three or four washes each with water, methanol, and chloroform–methanol, in that order. All washes were combined with the original sample and the lipids removed by evaporation under a stream of dry nitrogen. The lipids were then resuspended in exactly 1.0 mL of 2:1 (v/v) chloroform–methanol. Samples were analyzed for phosphate by using the Bartlett (1959) procedure and cholesterol by the enzymatic procedure of Moore et al. (1977).

A number of samples recovered from the calorimeter were examined for lipid breakdown by thin-layer chromatography on silica gel plates. No breakdown could be detected when the plates were developed in either 65:25:3.5:1.5 (v/v) chloroform–methanol–ammonia–water or 40:50:2:0.2 (v/v) ethyl ether–benzene–ethanol–acetic acid and the lipids visualized by iodine staining.

Results

Figures 1 and 2 display typical calorimeter scans of *N*-palmitoylsphingomyelin–cholesterol and *N*-lignocerylsphingomyelin–cholesterol mixtures, respectively. In Figure 3 is plotted the total enthalpy change of the endothermic peaks observed in these scans as a function of cholesterol content along with corresponding data from a previous study on dipalmitoylphosphatidylcholine (Estep et al., 1978). The temperatures of the various heat-capacity maxima as a function of cholesterol content for these phospholipids are presented in Figure 4.

In several respects the behavior of palmitoylsphingomyelin–cholesterol mixtures is reminiscent of that of dipalmitoylphosphatidylcholine–cholesterol mixtures. In both cases the total enthalpy change of the main endothermic peak in the calorimetric scans decreases as the relative cholesterol content increases, and in the presence of moderate amounts of sterol this peak consists of both sharp and broad components. In both cases the temperature of the sharp component maximum decreases as more cholesterol is added, while that for the broad component appears to increase. In the case of the palmitoylsphingomyelin, however, the decrease in the sharp component transition temperature is much more pronounced than with the phosphatidylcholine, giving a much better resolution of the two components. This is especially noticable in scans C, D, and E of Figure 1 where two maxima in the

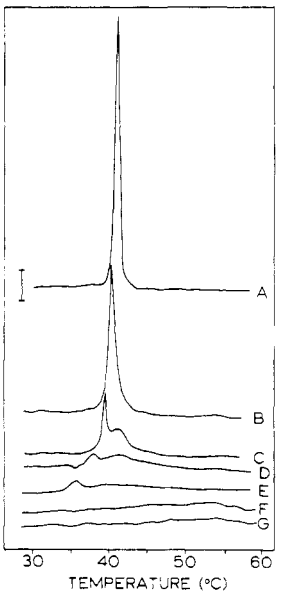

FIGURE 1: Calorimetric scans of aqueous dispersions of palmitoylsphingomyelin–cholesterol mixtures. Phospholipid concentration was approximately 10 mM in all samples. Heat-capacity values were calculated per mol of phospholipid. The vertical bar represents 1 kcal mol^{-1} deg^{-1}. Samples contained: (A) 0.0, (B) 6.2, (C) 11.6, (D) 15.6, (E) 19.3, (F) 26.0, and (G) 30.3 mol % cholesterol.

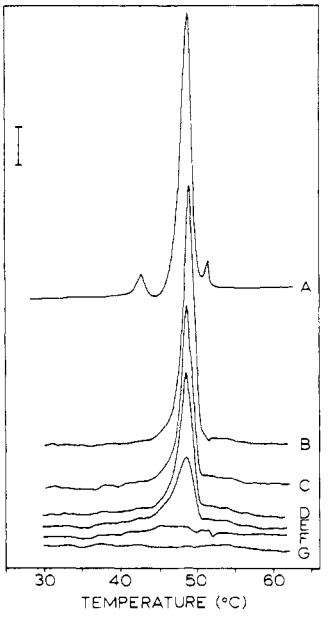

FIGURE 2: Calorimetric scans of aqueous dispersions of lignocerylsphingomyelin–cholesterol mixtures. Phospholipid concentration was approximately 10 mM in all samples. Heat-capacity values were calculated per mol of phospholipid. The vertical bar represents 1 kcal mol^{-1} deg^{-1}. Samples contained: (A) 0.0, (B) 6.9, (C) 12.4, (D) 15.0, (E) 20.1, (F) 24.4, and (G) 29.9 mol % cholesterol.

heat capacity function may be discerned. For scan E, this reflects the approximate 5 °C decrease in the palmitoylsphingomyelin sharp component maximum in the presence of 20 mol % cholesterol as opposed to a 1 °C decrease for the corresponding dipalmitoylphosphatidylcholine component with a similar amount of sterol.

A second difference between these two phospholipids is the manner in which the total enthalpy change is distributed between the sharp and broad components as a function of cholesterol content. This is shown in Figure 5 in which the enthalpy changes assignable to the separate components are plotted for both the sphingomyelin–cholesterol and phos-

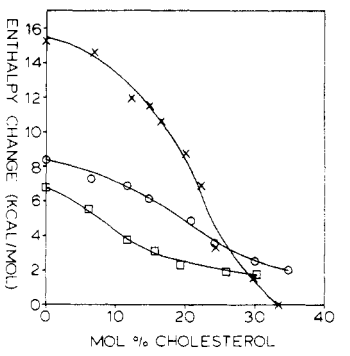

FIGURE 3: Effect of cholesterol on the total enthalpy change per mol of phospholipid of the gel to liquid-crystalline phase transition of palmitoylsphingomyelin (□), dipalmitoylphosphatidylcholine (○), and lignocerylsphingomyelin (X). Data for dipalmitoylphosphatidylcholine–cholesterol mixtures are taken from Estep et al. (1978). These data were calculated from the integral of heat-capacity plots such as those of Figures 1 and 2. The values for lignocerylsphingomyelin containing samples include contributions from the small endotherms at 42.5 and 51.2 °C.

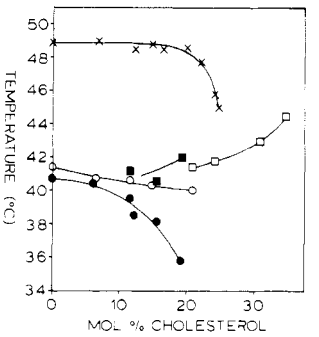

FIGURE 4: Temperatures of heat-capacity maxima vs. cholesterol content for aqueous dispersions of sterol with palmitoylsphingomyelin (●, sharp component; ■, broad component), lignocerylsphingomyelin (X), and dipalmitoylphosphatidylcholine (○, sharp component; □, broad component). Phosphatidylcholine values are taken from Estep et al. (1978).

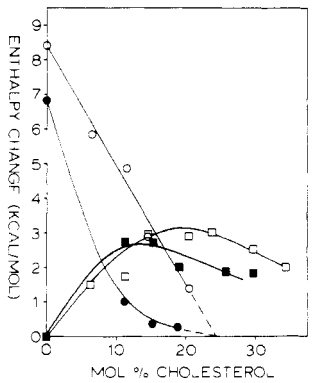

FIGURE 5: Resolution of the total endotherm enthalpy change into sharp and broad components as described under Results. The curves represent palmitoylsphingomyelin–cholesterol (sharp (●) and broad (■)) and dipalmitoylphosphatidylcholine–cholesterol (sharp (○) and broad (□)) components. Phosphatidylcholine data are taken from Estep et al. (1978).

phatidylcholine–cholesterol mixtures. The resolution of the total enthalpy change into separate components for the sphingomyelin–cholesterol suspensions was accomplished by noting that the broad component peak appears to be symmetric about a line drawn through its point of maximum heat capacity and perpendicular to the temperature axis. This permits construction of the low-temperature half of this component by reflecting the high-temperature portion about the symmetry axis. The area of the "pure" broad component so constructed

was subtracted from that of the total endotherm to give the area of the sharp component. It was then assumed that the ratio of a single component area to the total area was equal to the fraction of the total enthalpy change which could be associated with that particular component. This procedure was applied to all of the palmitoylsphingomyelin–cholesterol scans in which two heat capacity maxima were detectable. While these resolution procedures are undoubtedly somewhat arbitrary, we believe they provide useful estimates of the enthalpy changes associated with the two endotherm components.

The results of these analyses indicate that, whereas the enthalpy change of the sharp component approaches zero as the cholesterol content increases to 25 mol % for both palmitoylsphingomyelin and dipalmitoylphosphatidylcholine, the rate of decrease over this composition interval is linear for the latter phospholipid but nonlinear for the former. The palmitoylsphingomyelin–cholesterol sharp component enthalpy decreases more rapidly with an increase in sterol content than does the corresponding component in dipalmitoylphosphatidylcholine–cholesterol mixtures over the composition interval 0–10 mol % sterol, but less rapidly over the range 10–25 mol %, with the approach to zero apparent enthalpy being asymptotic in nature. On the other hand, the enthalpy changes ascribable to the respective broad components parallel one another closely except that the maximum with sphingomyelin containing mixtures occurs in the range 10–20 mol % cholesterol rather than in the 15–25 mol % range observed for the phosphatidylcholine mixtures. In both systems, the maximum broad component enthalpy is approximately 3 kcal/mol. Another feature common to both mixture types is the asymptotic approach of the broad component enthalpy to some, possibly nonzero, value as the cholesterol content is increased above 25 mol %; however, the extreme breadth of the endotherms at higher mol fractions of cholesterol makes it impossible to determine precisely this limiting value.

An additional point of comparison between palmitoylsphingomyelin–cholesterol and dipalmitoylphosphatidylcholine–cholesterol dispersions is the endotherm width at half peak height. In cholesterol–dipalmitoylphosphatidylcholine mixtures, the line width of the sharp component remains relatively constant at a value comparable to that of the pure phospholipid (0.4 ± 0.1 °C)[1] until the cholesterol content approaches 15 mol %. At approximately 20 mol % steroid, this component has broadened slightly to 0.6 ± 0.1 °C (T. N. Estep et al., unpublished results). When cholesterol is added to palmitoylsphingomeylin, the half-height width of the sharp component is approximately the same as that for the pure sphingolipid (0.6–0.9 °C) up to a sterol ratio of at least 11.6 mol %. In the presence of higher concentrations of cholesterol, there is noticable broadening, the peak width being 1.2 ± 0.1 °C with 15.6 or 19.3 mol % steroid. As noted above, the width of the broad component grows monotonically as the relative proportion of cholesterol is increased with both types of phospholipid. For palmitoylsphingomyelin, this change is from approximately 3 °C with 11.6 mol % cholesterol to greater than 10 °C at 26.0 mol %. Thus the peak component line widths follow the pattern of the other endotherm parameters

[1] A recent report by Albon & Sturtevant (1978) indicates that the true width at peak half-height of the main dipalmitoylphosphatidylcholine endotherm is less than 0.1 °C and that the larger values obtained in previous studies may be due to residual impurities. The sphingomyelins used in the present work may also contain such impurities so that the absolute value of the peak half-height width reported here may be in error. However, these considerations should not substantially alter the comparisons drawn here between the two phospholipid types.

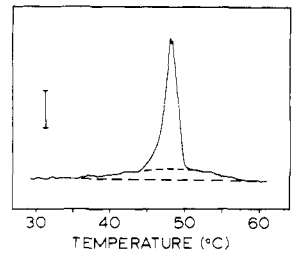

FIGURE 6: Example of sharp and broad component resolution for lignocerylsphingomyelin plus 15.0 mol % cholesterol. The vertical bar represents 1 kcal mol^{-1} deg^{-1}.

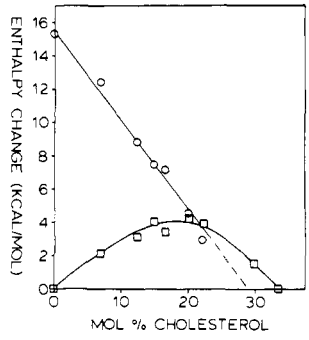

FIGURE 7: Resolution of the total endotherm enthalpy change of lignocerylsphingomyelin–cholesterol mixtures into sharp (○) and broad (□) components as described under Results.

in that they vary in a qualitatively similar manner as a function of cholesterol content for mixtures with either palmitoylsphingomyelin or dipalmitoylphosphatidylcholine.

A somewhat different set of circumstances is observed when cholesterol is codispersed with lignocerylsphingomyelin. From the scans displayed in Figure 2 and the data presented graphically in Figures 3 and 4, we see that, although the addition of cholesterol results in a decrease in the total endotherm enthalpy change and the appearance of a two component peak, the temperature of maximum heat capacity remains virtually constant at 48.7 ± 0.3 °C in mixtures containing up to 20 mol % sterol. It should be noted that repeat scans on mixtures containing lignocerylsphingomyelin are not as reproducible as those with palmitoylsphingomyelin or dipalmitoylphosphatidylcholine, a fact which is reflected in the greater variance in the observed temperature at which the heat-capacity maximum occurs.

The half-height width of the sharp endotherm was estimated after resolution of the total endotherm into sharp and broad components as described below. This width was constant at 1.7 ± 0.2 °C up to 16.6 mol % cholesterol and then broadened to 2.6 °C with 20.1 mol % sterol. The half-height width of the broad component was in excess of 10 °C at all concentrations of cholesterol.

The broader component, which extends over the temperature interval 35–60 °C, appears to have a heat-capacity maximum which falls underneath the sharper component so that two distinct heat-capacity maxima cannot be observed in these mixtures. Nevertheless, the two components can be resolved with a fair degree of accuracy because of the difference between their widths. The procedure utilized was to draw a reasonable connecting curve between the inflection points which consistently appear on either side of the transition, as shown for the sample containing 15.0 mol % cholesterol in Figure 6. The enthalpy of each component was then calculated as discussed above for the palmitoylsphingomyelin–cholesterol mixtures. The functional dependence of the component enthalpy changes on cholesterol content is illustrated in Figure 7. This procedure was not attempted on the

mixtures containing approximately 25 mol % cholesterol due to the ambiguity caused by the increasing breadth of the sharper component. The results indicate that the sharp component enthalpy change decreases with increasing cholesterol content, approaching zero between 25 and 30 mol % sterol, if one assumes a linear extrapolation. The broad component enthalpy change is maximal at approximately 20 mol % cholesterol and approaches zero as one decreases the sterol content to zero or increases it to 33 mol %. In this respect, the lignocerylsphingomyelin–cholesterol mixtures differ from those discussed previously in that the broad component enthalpy change at higher sterol concentrations does not asymptotically approach a limiting nonzero value but rather it reaches zero in a virtually linear fashion; however, it should be emphasized that this may only be an apparent effect due to an extremely rapid endotherm broadening with cholesterol concentrations above 25 mol %.

Two further observations concerning the effect of cholesterol on the calorimetric behavior of lignocerylsphingomyelin are warranted. The first is that no greater than 6.9 mol % cholesterol is required to abolish the small endotherms observed at 42.5 and 51.2 °C in scans of pure lignocerylsphingomyelin. The second is that the shape of the main endotherm exhibits increasing asymmetry as cholesterol is added, with a tailing toward the low-temperature side of the peak becoming more pronounced as the sterol ratio is increased.

Discussion

Pure sphingomyelins dispersed in excess aqueous solution exhibit a well-defined endotherm which is characteristic of a gel to liquid-crystalline phase transition (Barenholz et al., 1976). The results of the present study imply that this transition is altered in a rather complex fashion as increasing amounts of cholesterol are added to the system.

The most striking feature of the calorimetric scans of palmitoylsphingomyelin–cholesterol mixtures is the two distinct maxima in the endotherm. This behavior is strong evidence that these mixtures undergo phase separation, with the sharper endotherm corresponding to a gel to liquid-crystalline transition of a phase which is relatively enriched in sphingomyelin and the broader component associated in some manner with a phase enriched in cholesterol. This interpretation is based in part on the similarity between the calorimetric behavior of palmitoylsphingomyelin–cholesterol mixtures and dipalmitoylphospatidylcholine–cholesterol mixtures. For the latter system, a wealth of X-ray diffraction and spectroscopic evidence implies that phase separation occurs (Engelman & Rothman, 1972; Shimshick & McConnell, 1973; Phillips & Finer, 1974; Lee, 1976). This view of the nature of dipalmitoylphosphatidylcholine–cholesterol dispersions is further supported by the electron diffraction experiments of Hui & Parson (1975). It should, therefore, not be surprising to find that the structurally similar sphingomyelins also exhibit phase separation when mixed with cholesterol.

The sharp endotherm component in scans of palmitoylsphingomyelin–cholesterol dispersions is also identified as a sphingomyelin gel to liquid-crystalline transition on the basis of the observation that the shape of this peak and its temperature maximum are similar to those of the pure sphingomyelin. The progressive decrease in the enthalpy of the sharp component is due to the fact that, as more cholesterol is added, more phospholipid becomes incorporated into the sterol-rich phase and is, therefore, unable to undergo a sharp gel to liquid-crystalline transition. At some point, one would expect all of the sphingomyelin to be incorporated into the cholesterol-rich phase or an associated boundary region and, thus, for the endotherm of the phospholipid-rich phase to disappear. This occurs at approximately 25 mol % cholesterol for the palmitoylsphingomyelin–cholesterol system.

A distinct difference between palmitoylsphingomyelin and dipalmitoylphosphatidylcholine with respect to the presence of cholesterol is the greater initial decrease of the sharp component enthalpy upon sterol addition to the sphingomyelin. This difference suggests that the stoichiometric ratio of phospholipid to cholesterol in the sterol-rich phase is greater for palmitoylsphingomyelin than for dipalmitoylphosphatidylcholine.

The broad endotherm which is first resolvable at approximately 10 mol % sterol is probably present at much lower ratios of cholesterol. As was suggested for the corresponding component in dipalmitoylphosphatidylcholine–cholesterol mixtures (Estep et al., 1978), this peak can be explained either as a transition of cholesterol-rich domains directly or as a transition of the boundary regions surrounding such domains. The latter explanation is consistent with the observation that the broad component enthalpy change is maximal at 15 mol % cholesterol. As cholesterol is added to the system, the extent of boundary region increases until the relatively pure sphingomyelin phase disappears. Subsequent cholesterol addition causes a decrease in the extent of boundary region as the cholesterol-rich domains coalesce into larger units. It is assumed that, when two compositionally different phases coexist, they are finely dispersed so that the amount of lipid within boundary regions will be a significant fraction of the total lipid present. Electron diffraction (Hui & Parsons, 1975) and freeze-fracture electron microscopy (Gebhardt et al., 1977) experiments have shown this to be the case for phosphatidylcholine–cholesterol mixtures.

On the other hand, if the decrease in broad component enthalpy change is only an apparent effect resulting from the broadening of the transition, the assignment of this endotherm to a transition within the cholesterol-rich phase becomes more plausible. It is also possible that the broad endotherm is due to a cholesterol-rich phase of intermediate sterol to phospholipid which is subsequently supplanted by a phase more enriched in cholesterol. In this case, the decrease of broad endotherm enthalpy change above 15 mol % cholesterol would be attributable to the disappearance of the phase giving rise to this peak. If this interpretation is correct, the composition of the intermediate phase would correspond to the point of maximum broad component enthalpy change of 10–20 mol % sterol. Although with the information at hand a rigorous selection among the alternatives presented above is not possible, it is clear that phase separation occurs in palmitoylsphingomyelin–cholesterol mixtures.

The data for the lignocerylsphingomyelin–cholesterol mixtures can also be interpreted within the framework of the two-phase hypothesis. Thus, the addition of small amounts of cholesterol to lignocerylsphingomyelin causes the formation of cholesterol-rich domains leaving the rest of the sphingomyelin in a virtually pure state. This "pure" sphingomyelin can undergo a gel to liquid-crystalline phase transition which is essentially identical with that of the pure phospholipid. The decrease in sharp component enthalpy change is again readily explained as a diminution in the number of phospholipid molecules able to participate in the gel to liquid-crystalline transition. Classically, the occurrence of an isothermal transition in a binary mixture over a range of relative concentrations is taken as strong evidence of phase separation. Although the applicability of macroscopic thermodynamic rules to membrane systems has been questioned (Lee, 1975),

it is significant that lignocerylsphingomyelin–cholesterol mixtures exhibit phase separation by these criteria.

The behavior of the broad component of lignocerylsphingomyelin–cholesterol mixtures is interesting in several respects. The first is that it is quite broad even when first detectable, in contrast to the much narrower broad component seen in palmitoylsphingomyelin mixtures. While the exact significance of this is uncertain, it may indicate there are subtle differences in the interaction of cholesterol with these two sphingomyelin types. The second point with regard to the lignocerylsphingomyelin–cholesterol broad component is that it disappears in the presence of 33 mol % sterol. This is consistent with the existence of a cholesterol-rich phase of 2:1 sphingomyelin to sterol stoichiometry, whether this component is assumed to be a transition of a boundary region or of a cholesterol-rich phase containing a higher fraction of phospholipid. This interpretation assumes that no detectable transition arises directly from the 2:1 sphingomyelin–cholesterol phase.

The broadening of the palmitoyl and lignoceryl sharp components above 15 mol % cholesterol as well as the eventual decrease in the temperature of maximum heat capacity are most likely the result of a decrease in the average domain size of the pure sphingomyelin phase which causes a reduction in the extent of the average cooperative unit. This could also be the source of the more marked asymmetry observed in the lignocerylsphingomyelin sharp component over this composition interval.

In view of the present data, it is interesting to consider the results of experiments on mixtures of cholesterol with heterogeneous sphingomyelins. The calorimetric experiments reported by Demel et al. (1977) provide evidence for the existence of two transitions in mixtures of erythrocyte sphingomyelin with 10–20 mol % sterol, although the authors do not comment on this point. In addition, evidence has been presented for phase separation in beef brain sphingomyelin–cholesterol dispersions on the basis of fluorescence and enzymatic experiments (Cohen & Barenholz, 1978). It, therefore, appears that phase heterogeneity may be a common occurrence in membranes containing both sphingomyelin and cholesterol.

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