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Thermal Behavior of Stearoylsphingomyelin–Cholesterol Dispersions†

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ABSTRACT: The thermotropic behavior of aqueous dispersions of stearoylsphingomyelin–cholesterol mixtures was examined by high-sensitivity differential scanning calorimetry. When less than 20 mol % cholesterol was mixed with the sphingomyelin and the samples were held at room temperature for 7–9 days before the initiation of calorimetric measurements, a sharp endotherm at 56–57 °C and a broad endotherm at 35–50 °C were observed. In addition, samples containing 15–20 mol % stearol exhibited a sharp endotherm at 43–45 °C. If samples were held at room temperature for less than 2 h before the initiation of calorimetric analysis, the 56–57 °C endotherm was usually not seen. Instead, a combination of broad and sharp endotherms over the range of 35–50 °C was observed. Occasionally, exotherms were also observed within this temperature range. These results, along with those from previous studies, imply that a cholesterol-rich phase coexists with a cholesterol-poor phase in which the sphingomyelin molecules may exist in two distinctly different gel states.

Since sphingomyelin and cholesterol comprise a significant fraction of the lipids present in many mammalian cells (Rouser et al., 1968), the study of their mixtures is pertinent to the understanding of the structure of biological membranes. On the basis of chemical and physical evidence, it has been suggested that sphingomyelin and cholesterol exhibit a preferential interaction (Vandenheuvel, 1963; Patton, 1970; Demel et al., 1977). For these reasons a systematic investigation using differential scanning calorimetry of aqueous dispersions of mixtures of synthetic sphingomyelins with cholesterol was initiated. Results of experiments with sphingomyelins containing palmitoyl or lignoceryl fatty acyl residues have already been reported (Estep et al., 1979). Due to the unexpected behavior of dispersions containing stearoylsphingomyelin (Estep et al., 1980), studies of mixtures of this phospholipid with cholesterol are now being reported separately.

Experimental Procedures

Materials. DL-*erythro*-*N*-Stearoylsphingosinephosphorylcholine (stearoylsphingomyelin) was supplied by Professor D. Shapiro (Weizmann Institute of Science, Rehovot, Israel) and Dr. C. T. Schmidt (Department of Biochemistry, University of Virginia, Charlottesville, VA). The lipid was purified and analyzed as described previously (Estep et al., 1980). Only sphingomyelin which readily exhibited a single endotherm at 57 °C when dispersed into aqueous solution was utilized in

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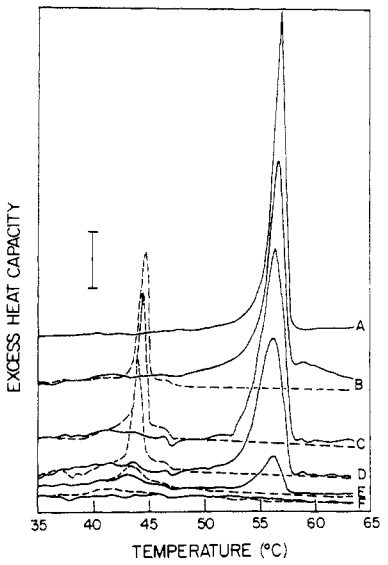

FIGURE 1: Calorimetric heating scans of aqueous dispersions of stearoylsphingomyelin–cholesterol mixtures. Phospholipid concentration was approximately 10 mM in all samples. Heat capacity values were calculated per mole of phospholipid. Scans performed after at least 7 days at room temperature are shown as solid lines. Dashed lines represent scans performed immediately after samples were cooled to room temperatures. The vertical bar represents 2 kcal $mol^{-1}deg^{-1}$. Samples contained (A) 0.0, (B) 1.1, (C) 5.1, (D) 11.0, (E) 16.8, and (F) 20.0 mol % cholesterol.

the formulation of mixtures with cholesterol in these experiments. Cholesterol was purchased and purified as described elsewhere (Estep et al., 1978).

Methods. The preparation of aqueous lipid dispersions, their subsequent chemical analysis, and the performance of differential scanning calorimetry were as outlined previously (Estep et al., 1979), except that dispersions were annealed for 1–2 h at 65 °C and then held at room temperature for time periods ranging from minutes to as long as 9 days prior to the initiation of calorimetric analysis. All dispersions were prepared in 50 mM KCl, and the lipid content was determined after the completion of the calorimetric measurements. The total lipid concentration in the dispersions was less than 2% (w/w) in all cases. All calorimetric data were acquired during increasing temperature scans.

Results

Preliminary calorimetric experiments with stearoylsphingomyelin–cholesterol mixtures indicated that the results obtained were highly dependent on sample history. On some occasions endothermic transitions were observed in the 56–57 °C and/or the 43–45 °C temperature interval along with an occasional exotherm in the 30–50 °C region. These results were highly reminiscent of the behavior reported previously when other hydrophobic compounds were mixed with stearoylsphingomyelin (Estep et al., 1980). As was the case in the earlier experiments, long incubations at room temperature prior to the initiation of calorimetric analysis favored the production of the state giving rise to a single endotherm at 57 °C, while short exposures to temperatures less than 57 °C resulted in the frequent appearance of endotherms at 43–45 °C. These observations prompted the performance of a series of experiments in which stearoylsphingomyelin–cholesterol dispersions were held for at least 7 days at room temperature before the first calorimetric scans were begun. Each sample was scanned from 30 to 65 °C following the room temperature incubation and then cooled as rapidly as possible (≃2 h) in the calorimeter to room temperature and a second scan immediately began.

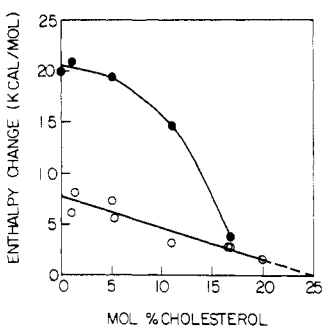

FIGURE 2: Effect of cholesterol on the total enthalpy of endotherms occurring in the 56–57 °C (●) and 35–50 °c (○) temperature intervals. The latter includes both sharp and broad components. The enthalpy changes are calculated per mole percent of total phospholipid and are calculated from the integral of heat capacity plots such as those of Figure 1.

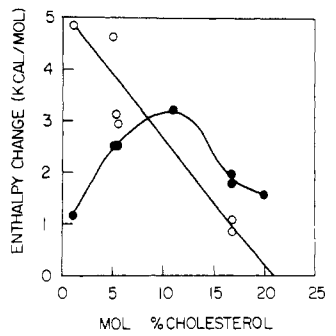

FIGURE 3: Resolution of the total enthalpy change of endotherms in the 35–50 °C temperature interval into sharp (○) and broad (●) components. The resolution of the individual components was performed as described in a previous publication (Estep et al., 1979).

In this series of experiments (Figure 1) samples containing up to 17 mol % cholesterol displayed a distinct 56–57 °C endotherm during the first scan but no transition in this region during the second. In contrast, a sharp endotherm in the 43–45 °C temperature interval was always evident in the second scan, but not the first, except for the sample containing 16.8 mol % cholesterol. In addition, there was a very broad endotherm over the 35–50 °C temperature interval evident in both the first and second scans of samples containing 1–17 mol % cholesterol. This broad endotherm was the only transition evident in scans of samples containing 20.0 mol % cholesterol. At higher mole fractions of cholesterol, no consistent deviation from the base-line heat capacity function was observed.

The enthalpy changes associated with the various endotherms were found to be strongly dependent on the cholesterol content of the sample (Figures 2 and 3). The total enthalpy associated with the sharp 56–57 °C and the sharp 43–45 °C endotherm decreased as the cholesterol content increased. On the other hand, the enthalpy change associated with the broad 35–50 °C endotherm increased with increasing cholesterol content up to approximately 11 mol % sterol and then decreased with increasing cholesterol content above this point. The data plotted in Figures 2 and 3 were derived from the scans illustrated in Figure 1 as well as from additional scans which are not shown. No data are included from experiments in which exotherms were present due to ambiguities in determining an appropriate base line. It should be noted that these results must be interpreted with caution since, as discussed below, some of the data are from samples which were not in thermodynamic equilibrium. In particular, samples which were incubated at room temperature for short periods of time were often not at equilibrium during the subsequent calorimetric measurements as suggested by the frequent ap-

pearance of exotherms under these conditions. Nevertheless, these data are useful in quantitating the observed behavior and in comparing this system to other phospholipid–cholesterol mixtures.

A significant aspect of the 56–57 °C endotherms and the sharp 43–45 °C endotherms is that these peaks exhibit only minor alterations in position and width as a function of cholesterol content. Over the range 0–17 mol % cholesterol, the heat capacity maximum of the higher temperature endotherm decreased from 57 to 56 °C, while that of the lower temperature endotherm declined by approximately 2 °C. The transition width at half peak height of the 56–57 °C endotherm increased from 1.2 °C to a maximum of 2.6 °C and that of the 43–45 °C endotherm increased from 1.0 to 2.6 °C as the mole fraction of cholesterol approached 17%. Thus, the shapes of the sharper endotherms were relatively insensitive to the cholesterol concentration, in contrast to the strong dependence of the enthalpy changes on this parameter. The shape and position of the broad 35–50 °C endotherm also appeared to be relatively unaffected by the cholesterol concentration, although the breadth of this component made it difficult to detect changes in these parameters.

Discussion

The results of the second scan of samples illustrated in Figure 1 are similar to those obtained from experiments with cholesterol–dipalmitoylphosphatidylcholine (Estep et al., 1978), –palmitoylsphingomyelin, or –lignocerylsphingomyelin mixtures (Estep et al., 1979). In each of these cases the addition of cholesterol resulted in a diminution in the total transition enthalpy change and the appearance of coexisting broad and sharp endotherms. The enthalpy change of the sharp components decreased monotonically in these mixtures as the cholesterol content was increased, while the enthalpy change associated with the broad endotherms initially increased as the mole fraction of sterol was raised, until a maximum in the apparent enthalpy change was attained at $\simeq$15 mol %. At higher cholesterol concentrations the enthalpy change of the broad component decreased as the sterol content was increased. The similarities in molecular structure and calorimetric behavior between stearoylsphingomyelin and the above-mentioned phospholipids suggest that the present results can be interpreted in the same manner as the results of earlier experiments.

In brief, it appears the admixture of cholesterol with these phospholipids results in the formation of a cholesterol-rich phase containing phospholipids which do not participate in the gel-to-liquid-crystalline phase transition typical of dispersions of the pure phospholipid. The broad endotherms seen in scans of these phospholipid–cholesterol mixtures appear to be associated, in some manner, with the cholesterol-rich phase, since the broad component is absent from calorimetric scans of pure phospholipid samples. The broad component in the heat capacity function of the mixtures most likely reflects the melting of those phospholipid molecules located at the boundaries of the cholesterol-rich domains (Estep et al., 1978; Snyder & Freire, 1980). The observed increase in the amplitude of the broad component at cholesterol mole fractions higher than 0.15–0.2 is consistent with this interpretation. The phospholipid molecules within the cholesterol-poor areas undergo the usual gel-to-liquid-crystalline phase transition seen with the pure phospholipid. This transition is observed in calorimetric scans as the sharp component of the excess heat capacity function. As the cholesterol content is increased, however, fewer phospholipid molecules are able to undergo the usual phase transition and the sharp endotherms are reduced in magnitude and eventually disappear. This occurs in the case of the stearoylsphingomyelin–cholesterol mixtures at 20–25 mol % cholesterol, the same mole fraction interval in which it occurs for dipalmitoylphosphatidylcholine– and palmitoylsphingomyelin–cholesterol mixtures. The identification of the sharp component as arising from a gel-to-liquid-crystalline phase transition of a relatively pure phospholipid domain is strongly supported in each case by the similarity in shape and t_m of the sharp component and the endotherm of the pure phospholipid.

The unusual feature of the stearoylsphingomyelin–cholesterol mixtures is the existence of two distinct sharp endotherms at 56–57 and 43–45 °C. An earlier study demonstrated that the pure phospholipid can exist in at least two different forms (Estep et al., 1980). The fully hydrated gel phase of pure stearoylsphingomyelin which is in thermodynamic equilibrium at room temperature melts to form a liquid-crystalline phase at 57 °C. This transition is not rapidly reversible upon cooling, however, with the sphingomyelin apparently forming a metastable gel which melts at 43–45 °C. This metastable gel form eventually reverts to the equilibrium form at room temperature in an exothermic process which is strongly influenced by the presence of hydrophobic impurities. Such impurities generally increase the lifetime of the metastable configuration. Thus, with samples incubated at room temperature for a week or more, the 56–57 °C endotherm was observed, whereas in samples which were exposed at room temperature for only a few hours, the 43–45 °C peak was predominant. The fact that some samples containing cholesterol displayed the sharp 43–45 °C endotherm even after a week at room temperature indicates that cholesterol, like other hydrophobic compounds, tends to prolong the existence of the metastable gel form of stearoylsphingomyelin. More significant, however, is the fact that the equilibrium gel state will form during a 1-week incubation at room temperature even in bilayers containing up to 17 mol % cholesterol. This suggests that over long time periods the 56–57 °C transition might be observable in even more complex mixtures containing greater amounts of cholesterol. The possible existence of at least two phases in phospholipid–cholesterol mixtures is supported by results from a number of laboratories (Engelman & Rothman, 1972; Shimshick & McConnell, 1973; Phillips & Finer, 1974; Hui & Parsons, 1975; Lee, 1976). The unusual aspects of the stearoylsphingomyelin experiments reported here tend to substantiate this concept.

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Modification by Papain of the Structure and Function of Band 3, the Erythrocyte Anion Transport Protein†

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ABSTRACT: Extracellular papain is known to inhibit the anion transport function of the band 3 protein of the human red blood cell membrane. Previous work [Jennings, M. L., & Passow, H. (1979) *Biochim. Biophys. Acta 554*, 498–519] had suggested that this inhibition may result from the removal by papain of 5000–10 000 daltons from the 35 000-dalton chymotryptic peptide of band 3. The present work shows, however, that papain also removes a small peptide from the C terminus of the 60 000-dalton chymotryptic peptide. The C-terminal amino acid sequence of this peptide is -Lys-Thr-Tyr. Whether or not this newly discovered action of papain is responsible for inhibiting anion transport is unknown. The effects of extracellular papain on the band 3 function have been characterized in detail. Papain inhibits Cl–Cl exchange in a high Cl medium by almost 90%. This inhibition appears to result from inhibition of the efflux step in the catalytic cycle for the transport, because papain does not inhibit the anion transport when it is assayed under influx-limited conditions. Moreover, since papain has no detectable effect on the dissociation constant for extracellular substrate (SO_4) binding, the material removed by papain cannot be involved closely in the outward-facing substrate site. In contrast, removal of this material strongly (12-fold) reduces the affinity of the inhibitor 4,4′-dinitro-2,2′-stilbenedisulfonate for outward-facing sites. Therefore, stilbenedisulfonate binding involves portions of the band 3 molecule which are not intimately related to substrate binding.

Band 3 (Fairbanks et al., 1971) is the major integral membrane protein of the human red blood cell. It has a subunit molecular weight of about 95 000 and functions in the catalysis of anion transport (Cabantchik & Rothstein, 1974a; Passow et al., 1975; Ho & Guidotti, 1975), the physiological mode of which is Cl–HCO_3 exchange. Despite recent advances in the understanding of both the structure and function of band 3 [see Steck (1978) and Knauf (1979)], very few relationships between structure and function have been established.

Analysis of the products of in situ enzymatic proteolysis of band 3 is a potential source of structure–function information. The advantages of in situ proteolysis are that the sidedness of the action of the enzyme on band 3 can be controlled, proteolysis is limited to a small number of sites, and the transport function of the protein can be assayed after the proteolysis. Since band 3 is a major component of the membrane, large cleavage products are relatively easy to recover and isolate.

It is well established that extracellular chymotrypsin cleaves band 3 into two peptides, both of which remain firmly attached to the membrane (Cabantchik & Rothstein, 1974b; Steck et al., 1978; Markowitz & Marchesi, 1981). These peptides have approximate molecular weights of 60 000 and 35 000, and they very likely remain associated with each other in a native complex after the proteolysis (Jennings & Passow, 1979; Reithmeier, 1979). Chymotrypsin treatment does not affect the transport function of the protein. In contrast, extracellular papain strongly inhibits both Cl–Cl and SO_4–SO_4 exchange (Passow et al., 1977; Ku et al., 1979). Jennings & Passow (1979) showed that this inhibition may be related to the degradation by papain of the 35 000-dalton chymotryptic peptide, although removal of material (less than or equal to six residues) from the 60 000-dalton peptide could not be ruled out.

The present results show that papain does in fact remove a small peptide from the 60 000-dalton peptide and that the elementary kinetic step which is inhibited by papain is anion efflux. We also demonstrate that papain does not alter substrate (SO_4) binding affinity at the extracellular surface but that it does strongly reduce the affinity for the disulfonic stilbene inhibitor 4,4′-dinitro-2,2′-stilbenedisulfonate (DNDS).[1]

Materials and Methods

Chemicals. Sources of reagents were the following: enzymes from Boehringer Mannheim, Indianapolis, IN; PMSF (phenylmethanesulfonyl fluoride), Sephadex G10, and Sepharose 6B from Sigma, St. Louis MO; $^{35}SO_4$ from New England Nuclear, Boston, MA; ^{36}Cl from ICN Radiochemicals, Irvine, CA; DIDS (4,4′-diisothiocyano-2,2′-stilbenedisulfonate) from Pierce, Rockford, IL. The dihydro analogue of DIDS (H_2DIDS) was a generous gift of Professor H. Passow. DNDS (technical; Aldrich, Milwaukee, WI) was purified by adsorption to Sephadex G10 in aqueous 100 mM Na_2SO_4, followed by desorption with distilled water.

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[1] Abbreviations used: DNDS, 4,4′-dinitro-2,2′-stilbenedisulfonate; DIDS, 4,4′-diisothiocyano-2,2′-stilbenedisulfonate; H_2DIDS, 4,4′-diisothiocyanodihydrostilbene-2,2′-disulfonate; PMSF, phenylmethanesulfonyl fluoride; BSA, bovine serum albumin; CPA, carboxypeptidase A; Na-DodSO$_4$, sodium dodecyl sulfate; EDTA, ethylenediaminetetraacetic acid; Cl_3CCOOH, trichloroacetic acid.

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