label is considered. This lower incorporation of the C-1 of glycine is believed to result from a selective loss of the C-1 of the glycine during the glycine cleavage reaction. The reversible reaction decomposes glycine to form 1 mol of methylene-H_4folate, ammonia, and CO_2 and has been demonstrated in *E. coli* as well as other bacteria (Pitts & Crosbie, 1962; Newman & Magasanik, 1963). Glycine formed from the reverse of this reaction would thus retain only the C-2 carbon. Considering the difference in the incorporation of the C-1 and C-2 carbons of the glycine, it was calculated that 15.5% of the cellular glycine must have been equilibrated in this reaction. Since the nitrogen would also be lost in this reaction, this then leaves only 9.8% (100 − 74.7 − 15.5%) of the cellular glycine to be produced via transamination or total synthesis. From the isotopes incorporated into the fragment ions of the pyrimidine, it is clear that the C-1 of the labeled glycine is incorporated at C-4 of the pyrimidine ring. This is consistent with the work of Estramareix & Lesieur (1969), who have shown that the C-1 of glycine supplies the C-4 carbon of the pyrimidine in *S. typhimurium*.

Considering that both of the carbons and the nitrogen of glycine are incorporated with no dilution from the metabolic pool of glycine present in the cell, it is clear that all of these atoms must be incorporated as a unit into the pyrimidine. These results are consistent with the work of Newell & Tucker (1968), who have shown that 4-aminoimidazole ribonucleotide is an intermediate in the biosynthesis of the pyrimidine moiety of thiamin in *S. typhimurium*. This follows from the established fact that both carbons and nitrogen of glycine are incorporated as a unit in the biosynthesis of the 4-aminoimidazole portion of the ribonucleotide.

The lack of incorporation of deuterium from the deuterated glycine is also consistent with AIR being an intermediate in pyrimidine biosynthesis. The loss of deuterium can be accounted for by the reversible carboxylation of AIR to CAIR during purine biosynthesis (Litchfield & Shaw, 1971). This reaction would result in the complete loss of the glycine deuterium for each molecule of AIR run through the carboxylation. The fact that some of the glycine deuterium is incorporated into the final C-6 position of the pyrimidine indicates that deuterium on the C-4 of AIR must not be completely lost during its conversion to the pyrimidine.

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Stabilizing Effect of Cholesterol on Phosphatidylcholine Vesicles Observed by Ultrasonic Velocity Measurement†

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ABSTRACT: The temperature dependence of the ultrasonic velocity was measured in sonicated vesicles of dipalmitoylphosphatidylcholine by varying the content of cholesterol. When cholesterol is incorporated, an anomalous dip of the ultrasonic velocity gradually smeared out. At the same time, the ultrasonic velocity of the membrane increased remarkably above 30 °C due to the increase of the bulk modulus by about 15%. On the other hand, the ultrasonic velocity and the bulk modulus decreased below 30 °C. Comparing the cholesterol-incorporated membrane with vesicles of bovine brain sphingomyelin and human erythrocyte membrane, we discuss the role of cholesterol in biological membranes in terms of the stability of the membrane as a barrier.

Cholesterol is one of the major components in biological membranes, yet the role of cholesterol in membranes is not clear enough. Recent investigations by model membranes, however, have elucidated the effects of cholesterol on various physicochemical properties of membranes. The effects of cholesterol may be summarized into two kinds of phenomena. One is the broadening of the phase transition, as shown by thermal analysis (Ladbrooke et al., 1968; Hinz & Sturtevant, 1972; Estep et al., 1978), Raman spectroscopy (Lippert & Peticolas, 1971), and permeability measurements (Blok et al., 1977). Another is the dual effect on the fluidity of membranes (Oldfield & Chapman, 1972a,b; Rothman & Engelman, 1972), that is, the rigidizing effect in the liquid crystalline phase and the fluidizing effect in the gel phase (Lippert & Peticolas, 1971; Kawato et al., 1978). These effects of cholesterol are directly related to the barrier properties of membranes rather than to some specific membrane functions. Therefore, it appears that the biological function of cholesterol is concerned with the stability of the membrane as a barrier (Rothman & Engelman, 1972; Oldfield & Chapman, 1971).

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The ultrasonic measurements have been applied recently to the suspension of lipid vesicles, and the ultrasonic velocity and attenuation have provided valuable information about the mechanical properties of membranes in the transition regions. Anomalous increase of the sound attenuation (Eggers & Funck, 1976) and decrease of the ultrasonic velocity have been found in the vicinity of the transition temperature of synthetic phosphatidylcholine membranes and considered to represent a distinct fluctuation in the membrane (Mitaku et al., 1978). Moreover, the permeability of a membrane is found to be in good correlation with the bulk modulus K (Mitaku et al., 1978) which may be obtained from the ultrasonic velocity according to eq 1 where V and ρ are the ultrasonic velocity and the

$$V = (K/\rho)^{1/2} \tag{1}$$

density, respectively. Therefore, the ultrasonic velocity has to be a good measure of the stability of a membrane as a barrier.

In this work, we present evidence from the ultrasonic velocity measurements indicating that the effect of cholesterol is to stabilize the membrane particularly in the liquid-crystalline phase. In order to discuss the biological role of cholesterol, the results for DPPC–cholesterol[1] membranes are compared with the vesicles of sphingomyelin from bovine brain and with the erythrocyte membranes.

Experimental Section

Materials and Preparation of Vesicles. Synthetic L-α-dipalmitoylphosphatidylcholine was purchased from Sigma Chemical Co. Cholesterol and bovine brain sphingomyelin were the products of Tokyo-kasei Co. No major contaminants were detected by thin-layer chromatography, and the lipids were used without further purification.

Sonicated small vesicles were prepared in order to avoid the sedimentation of lipid vesicles during experiments. The suspension of vesicles was prepared as described previously (Mitaku et al., 1978). DPPC was dissolved in chloroform together with cholesterol at the desired molar ratio. The solution was evaporated to dryness under vacuum, followed by the addition of the solvent, 150 mM NaCl with a phosphate buffer of pH 7.0. Then the sample was subjected to ultrasonic irradiation for 15 min at the power level of 150 W. Vesicles prepared by this method did not sediment during experiments of about 15 h. The weight concentration was measured by drying the sample at 110 °C under vacuum for a day.

Measurements of Ultrasonic Velocity and Density. The ultrasonic velocity was measured at 3 MHz by a differential ultrasonic velocimeter which was developed by Mitaku & Sakanishi (1977). The difference in the ultrasonic velocity is directly measured between a sample and the solvent that are contained in twin cells. Accuracy of the difference in the ultrasonic velocity is about ±0.7 cm/s, and the long-term stability is ±0.7 (cm/s)/day. The temperature dependence of the ultrasonic velocity was measured after a temperature equilibration of 20 min for each point.

The vibrational densitometer which was used in this work consists of a U-shaped capillary tube whose resonance frequency of vibration is about 300 Hz. The density of the liquid is determined from the change in the frequency of vibration due to the charging of the sample liquid into the capillary. We have carefully calibrated the apparatus constants against temperature, and experiments of temperature scanning became possible with the accuracy of about 50 ppm. The details of the apparatus and calibration will be described elsewhere.

Derivation of Bulk Modulus of Membrane. In order to determine the bulk modulus and the density of membranes from the ultrasonic velocity and the density measurements of suspensions, we define the limiting numbers of ultrasonic velocity $[V]$, density $[\rho]$, and bulk modulus $[K]$ as

$$[V] = \lim_{c \to 0} \frac{V - V_0}{V_0 c} \tag{2}$$

$$[\rho] = \lim_{c \to 0} \frac{\rho - \rho_0}{\rho_0 c} \tag{3}$$

$$[K] = \lim_{c \to 0} \frac{K - K_0}{K_0 c} \tag{4}$$

in which c is the dry weight concentration of the membrane and the subscript 0 denotes the solvent. Since $(V - V_0)/V_0$, $(\rho - \rho_0)/\rho_0$, and $(K - K_0)/K_0$ are much smaller than unity in a dilute membrane suspension, the following relation between the limiting numbers is easily derived from eq 1.

$$[V] = \tfrac{1}{2}([K] - [\rho]) \tag{5}$$

We can determine $[V]$ and $[\rho]$ from the measurements of the ultrasonic velocity and the density in suspensions, and then $[K]$ is calculated by eq 5. The limiting numbers of density and bulk modulus are described by eq 6 and 7, which are

$$[\rho] = \frac{\rho_m - \rho_0}{\rho_0} \frac{1 + \delta}{\rho_m} \tag{6}$$

$$[K] = \frac{K_m - K_0}{K_m} \frac{1 + \delta}{\rho_m} \tag{7}$$

equivalent to the additivity of the compressibility $1/K$ and the density ρ (Sakanishi et al., 1976) where ρ_m, K_m and δ are the density, the bulk modulus, and the amount of the hydrated water of the membrane, respectively. The term $(1 + \delta)/\rho_m$ is a normalization factor of the concentration. Assuming a reasonable value of δ, 0.5, we calculated the density and the bulk modulus of membranes by using eq 6 and 7 (Mitaku et al., 1975; Mitaku et al., 1978).

Results

The difference in the ultrasonic velocity ΔV between a vesicle suspension and the solvent was measured in the temperature range from 0 to 55 °C. DPPC vesicles with cholesterol of 0, 14.3, 32.3, and 49.0% in molar fraction were used for the measurements, and the dry-weight concentration of these samples was 1.79, 2.92, 4.23, and 6.09 mg/mL, respectively. Since ΔV linearly depends on the concentration in this concentration range, the limiting velocity number was calculated simply by dividing ΔV with the dry-weight concentration of the vesicles. Figure 1 shows the effect of cholesterol incorporation on the temperature dependence of the limiting velocity number of these mixed vesicles. The very sharp transition of the pure compound undergoes remarkable changes by the addition of cholesterol with respect to the anomalous dip in the transition region and also to the quantitative value of the limiting velocity number. The transition temperature T_c of pure DPPC vesicles is found to be 42 °C, and the anomalous dip which represents a critical fluctuation in the order of the membrane (Mitaku et al., 1978) is observable in the temperature region of $|T - T_c| < 8$ °C. When cholesterol is incorporated, the dip of the ultrasonic velocity gradually smears out until it completely disappears at the cholesterol content of about 50%. Upon the cholesterol incorporation of 14.3%, the dip becomes distinctly shallower

[1] Abbreviations used: DPPC, dipalmitoylphosphatidylcholine.

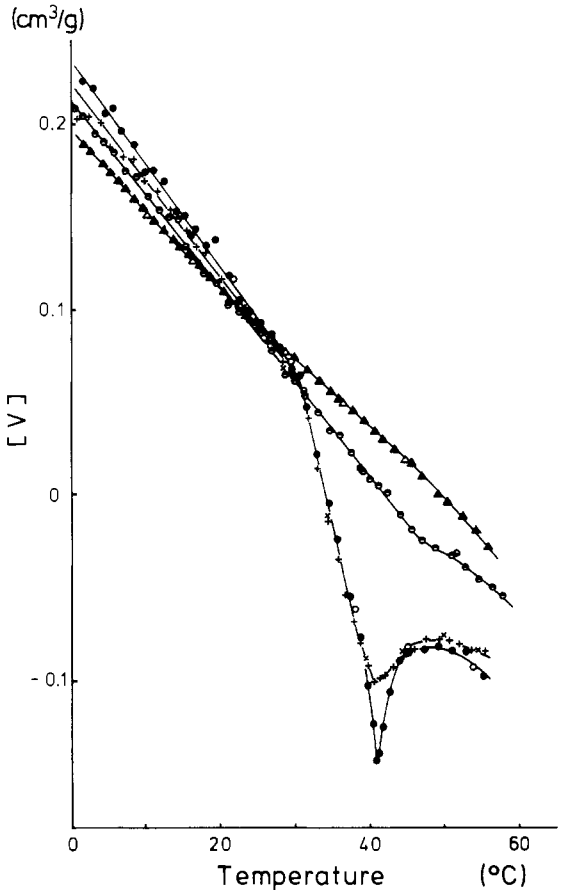

FIGURE 1: Temperature dependence of the limiting velocity number of sonicated vesicles prepared from dipalmitoylphosphatidylcholine and cholesterol. Molar fraction of cholesterol is 0 (●, ○), 14.3 (+, ×), 32.3 (◒, ◓), and 49.0% (▲, △).

in the temperature region $|T - T_c| < 2$ °C, while the ultrasonic velocity remains almost unchanged in other temperature ranges. On the other hand there is little indication of anomaly at the cholesterol content of 32.3% except for a slight break of the temperature dependence at 47 °C, and the membrane containing cholesterol of 49.0% does not show any transitional anomaly.

The limiting velocity number increases remarkably by the incorporation of cholesterol above the pretransition temperature of about 30 °C, while it decreases slightly below 30 °C. These facts are consistent with the so-called dual effect of cholesterol (Lippert & Peticolas, 1971; Oldfield & Chapman, 1972a,b; Rothman & Engelman, 1972). However, the magnitude of the variation in the limiting velocity number is larger above 30 °C than it is below 30 °C. Namely, the rigidizing effect in the liquid-crystalline phase appears to be more profound than the fluidizing effect in the gel phase.

Although the cholesterol is successively increased by about 15%, the change in the limiting velocity number is not proportional to the cholesterol content. The limiting velocity number is shown in Figure 2 as a function of cholesterol content. It appears that above 30 °C the ultrasonic velocity abruptly changes between 15 and 30% in cholesterol content, while the change in $[V]$ is very small below 30 °C throughout the cholesterol content under investigation. There may be some critical content of cholesterol to suppress the fluctuation of hydrocarbon-chain ordering.

The density of DPPC vesicles containing cholesterol of 49.0% was also measured by a vibrational densitometer. The limiting density number, $[\rho]$, of this sample is shown in Figure

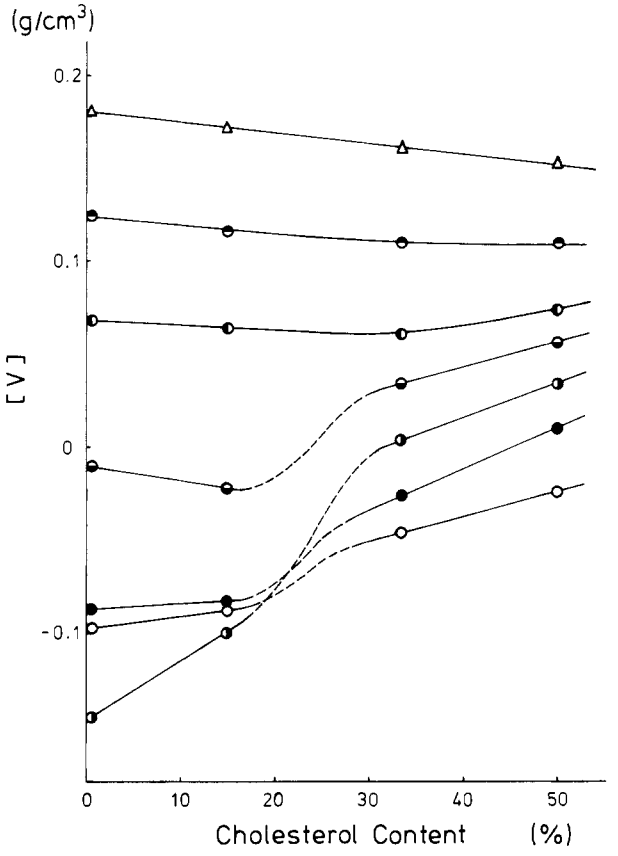

FIGURE 2: Limiting velocity number of the DPPC membrane is plotted against cholesterol content for the temperatures of 10 (△), 20 (◓), 30 (◐), 35 (◒), 42 (◑), 45 (●), and 55 °C (○).

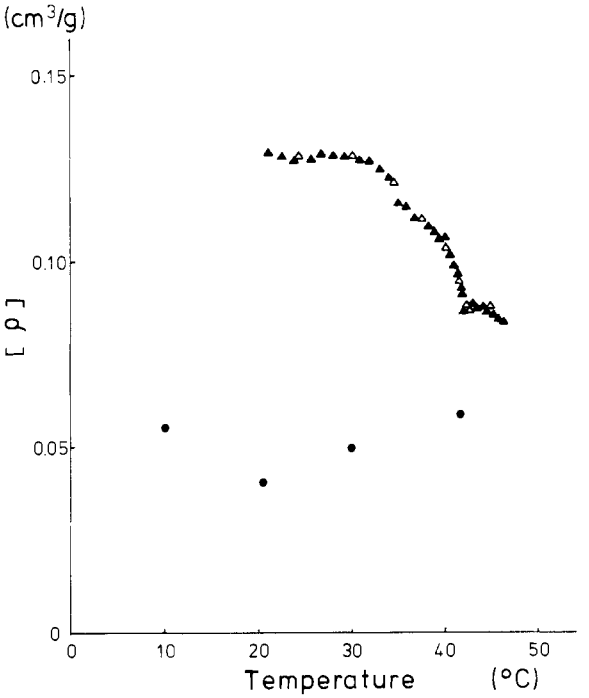

FIGURE 3: Limiting density number of sonicated dipalmitoylphosphatidylcholine vesicles with 49.0% cholesterol (●) and without cholesterol (▲, △).

3 together with the result in pure DPPC vesicles which has been reported by Mitaku et al. (1978). The limiting density number of DPPC–cholesterol vesicles is about 0.05 cm³/g and almost constant from 10 to 42 °C. The value of $[\rho]$, 0.05 cm³/g, is smaller than that of pure DPPC vesicles. Therefore,

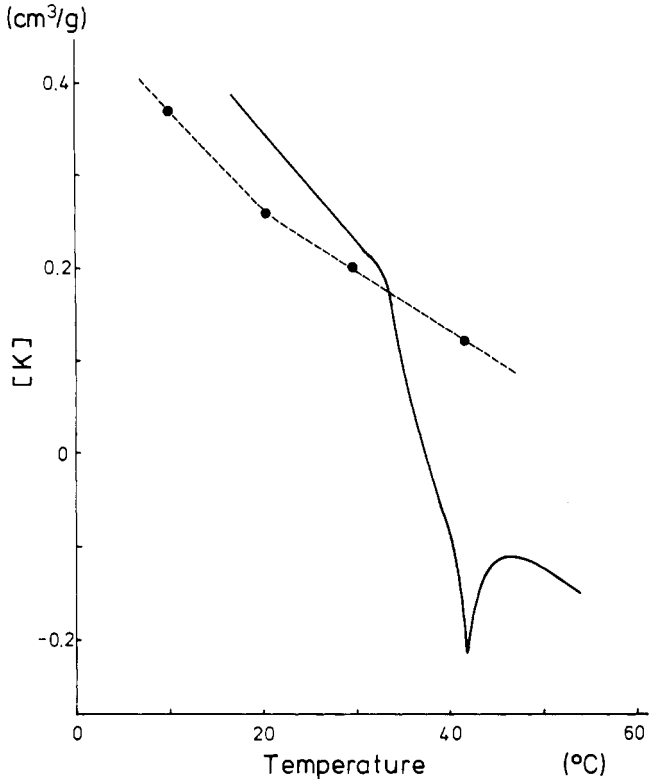

FIGURE 4: Limiting number of bulk modulus of sonicated dipalmitoylphosphatidylcholine vesicles with 49.0% cholesterol (●) and without cholesterol (—).

the DPPC membrane containing equimolar cholesterol presumably has a larger vacancy in the lipid bilayer than the DPPC membrane without cholesterol.

In order to estimate the bulk modulus of the membrane, the limiting number of bulk modulus, $[K]$, was calculated from $[V]$ and $[\rho]$ according to eq 5. Figure 4 shows $[K]$ of the sonicated vesicles in the presence and absence of cholesterol. The result indicates that upon the cholesterol incorporation the bulk modulus markedly increases above the pretransition temperature and decreases below it. This fact proves that cholesterol makes the membrane harder in the liquid-crystalline phase and softer in the gel phase against a bulk deformation. Contrary to cholesterol-free vesicles, the limiting number of bulk modulus of cholesterol-incorporated vesicles does not become negative in the temperature range between 0 and 50 °C. Namely, the bulk modulus of the membrane is always larger than that of the solvent.

Assuming that $\delta = 0.5$, we calculated the bulk modulus and the density of DPPC–cholesterol membranes from Figures 3 and 4 according to eq 6 and 7. The hydration of $\delta = 0.5$ is considered to be a reasonable value, and the variation in δ does not affect the results as much (Mitaku et al., 1978). The bulk modulus and the density of the DPPC membrane in the presence of cholesterol of 49.0% is compared in Table I to those of the pure DPPC membranes. Since the anisotropy of the elastic constant of the membrane is small (Le Pesant et al., 1978), the calculated values of the bulk modulus well represent the average modulus of the membrane. The pure DPPC membrane changes its bulk modulus from 2.95×10^{10} to 2.2×10^{10} dyn/cm^2 through an anomalous dip at about 42 °C, and the density decreases abruptly by about 5% at the transition temperature. On the other hand, the bulk modulus of membrane containing cholesterol does not show any anomalous change but decreases monotonously from 2.88×10^{10} to 2.50×10^{10} dyn/cm^2. Namely, upon the incorporation

Table I: Effect of Cholesterol on the Density and the Bulk Modulus of Sonicated DPPC Vesicles

	density (g/cm³)			bulk modulus (10^{10} dyn/cm²)		
temp (°C)	DPPC	DPPC–cholesterol	solvent	DPPC	DPPC–cholesterol	solvent
10		1.045	1.0067		2.88	2.14
20	1.099	1.033	1.0049	2.95	2.73	2.24
30	1.096	1.037	1.0021	2.77	2.68	2.31
42	1.060	1.039	0.9978	2.07	2.59	2.37
50	1.048	1.041	0.9947	2.20	2.50	2.39

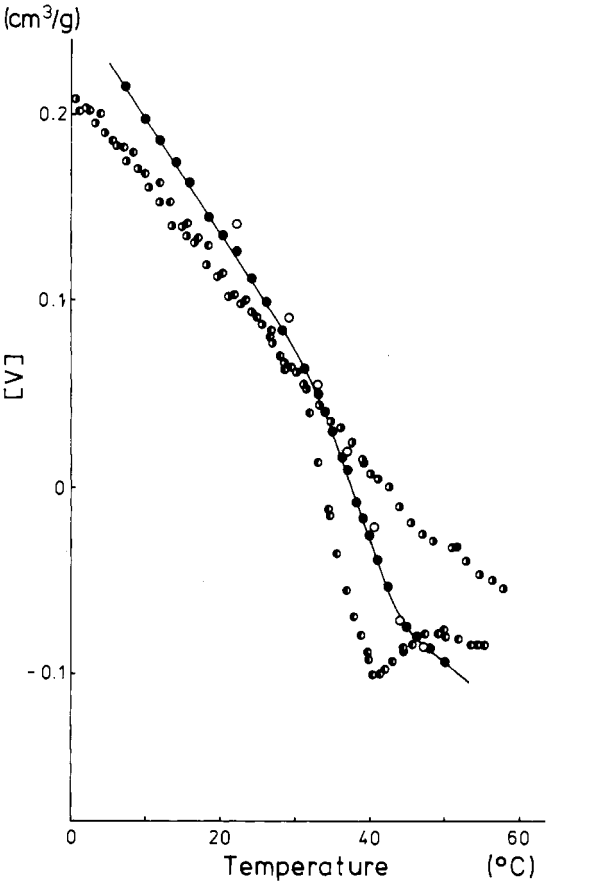

FIGURE 5: Limiting velocity number of sonicated vesicles of bovine brain sphingomyelin (●, ○) is compared with dipalmitoylphosphatidylcholine with 14.3 (◐) and 32.3% (◑) cholesterol.

of cholesterol the bulk modulus of the membrane decreases from 2.95×10^{10} to 2.73×10^{10} dyn/cm^2 at 20 °C, while it increases from 2.20×10^{10} to 2.50×10^{10} dyn/cm^2 at 50 °C. The change is largest at the transition temperature and amounts to 25%. The density of the cholesterol-incorporated membrane is almost constant from 10 to 50 °C and smaller than that of pure DPPC membrane, indicating that there is larger vacancy in the cholesterol-incorporated membrane.

The ultrasonic velocity is also measured in a suspension of sonicated vesicles of bovine brain sphingomyelin, whose hydrocarbon-chain length is heterogeneous (Shinitzky & Barenholz, 1974) and polar group is the same as that of phosphatidylcholine. The limiting velocity number of sphingomyelin vesicles is compared in Figure 5 with that of DPPC vesicles containing cholesterol of 14.3 and 32.3%. The limiting velocity number of sonicated sphingomyelin vesicles changes its temperature coefficient between 34 and 43 °C, corresponding to a phase transition observed by calorimetry (Oldfield & Chapman, 1972a; Barenholz et al., 1976) and NMR (Schmidt et al., 1977). It appears that the temperature

dependence of the ultrasonic velocity in sphingomyelin vesicles falls between the DPPC vesicles with cholesterol of 14.3 and 32.3% in their shape.

Discussion

The ultrasonic velocity in the sonicated vesicles of pure DPPC has a distinct minimum at the main transition temperature of 42 °C and a break at the pretransition temperature of 34 °C. Suurkuusk et al. (1976) have suggested from the fluorescence depolarization measurements that the transition temperature of the sonicated sample shifts to lower temperatures. However, the minimum of the ultrasonic velocity apparently remains at about 42 °C. This disagreement presumably arises from the fact that the ultrasonic propagation is sensitive to the fluctuation of the long-range order (Garland, 1970) whereas the fluorescence anisotropy varies according to the average order of the lipid bilayer. Therefore, the results of the present work and Suurkuusk et al. (1976) indicate that the order of hydrocarbon chains disappears and its fluctuation has a maximum at about 42 °C in sonicated small vesicles. Since the transition point of a higher order transition is generally defined as the temperature at which the order parameter vanishes and the fluctuation is largest, the main transition temperature of the sonicated vesicles is probably about 42 °C (Mitaku et al., 1978).

The phase transition of DPPC membrane smears out by the incorporation of cholesterol. When cholesterol of 14.3% is incorporated, the anomalous dip in the vicinity of the transition temperature becomes considerably shallower. Vesicles with cholesterol of 32.3% show only a slight break at about 47 °C. As mentioned above, an anomalous dip of the ultrasonic velocity in the DPPC membrane is caused by a critical fluctuation in the vicinity of the transition temperature; long-range order of hydrocarbon-chain packing is fluctuating in the temperature range between 34 and 50 °C. Therefore, the disappearance of the anomalous dip by the cholesterol incorporation indicates a reduction of the critical fluctuation of the hydrocarbon-chain ordering. Namely, the ordered as well as disordered domains of hydrocarbon chains probably do not grow large due to the steric effect of cholesterol.

Hinz & Sturtevant (1972) have reported that the transition enthalpy is a linear function of cholesterol content. Estep et al. (1978) have also shown that the sharp component of the transition enthalpy linearly decreases with the increase of the cholesterol content. However, Figure 2 indicates that the transition anomaly of the ultrasonic velocity changes sharply at the cholesterol content between 14.3 and 32.3%, suggesting some critical content of cholesterol effect. This difference may be due to the difference of the preparation of vesicles. Hinz & Sturtevant (1972) and Estep et al. (1978) have used nonsonicated liposomes, while the sonicated vesicles are employed in the present work. As far as the transition anomaly is concerned, the sonicated vesicles are very different from the nonsonicated liposomes (Suurkuusk et al., 1976); the first-order character of the transition in nonsonicated liposomes disappears by the preparation of sonicated vesicles (Mitaku et al., 1978). Therefore, sonicated vesicles do not have to show the same dependence on the cholesterol content as nonsonicated liposomes. However, a more plausible cause of this inconsistency seems to be that the calorimetry measures the latent heat just at the transition temperature, while the ultrasonic anomaly is due to the critical phenomenon in the vicinity of the transition temperature. In fact, the critical cholesterol content suggested from Figure 2 is distinct in a wide temperature region between 35 and 50 °C. The result of calorimetry, therefore, suggests that the latent heat of the transition linearly decreases with the incorporation of cholesterol. In contrast, the ultrasonic velocity measurement shows that the suppression of the critical fluctuation probably occurs only when the cholesterol content exceeds some value between 14.3 and 32.3%. More detailed study is necessary, however, to prove this suggestion definitely.

There has been some scattering of data with respect to the cholesterol content at which transition enthalpy vanishes. Ladbrooke et al. (1968) have reported that the endothermic peak disappears when equimolar cholesterol is included in the DPPC membrane. On the other hand, Hinz & Sturtevant (1972) have suggested that the cholesterol incorporation of 33% is sufficient for the removal of the phase transition. Intimate analyses by Estep et al. (1978) have recently elucidated that the transition enthalpy is finite even at 35%. The small break of the ultrasonic velocity in the vesicles containing cholesterol of 32.3% appears to be consistent with the results by Estep et al.

As shown in Table I and Figure 4, DPPC membrane in the gel phase becomes softer and disordered membrane becomes harder against a bulk deformation by the incorporation of cholesterol, which is consistent with the measurement of molecular mobility in the membrane. Oldfield & Chapman (1972a,b) have shown that by the incorporation of equimolar cholesterol the mobility of hydrocarbon chains is enhanced in the gel phase, while it is reduced in the liquid-crystalline phase. Lippert & Peticolas (1971) have studied the vibrational mode of hydrocarbon chains by Raman spectroscopy and also found the dual effect of cholesterol. The present measurement of the ultrasonic velocity has elucidated that not only the mobility of individual molecules but also a macroscopic mechanical property like bulk modulus is subject to the dual effect of cholesterol.

Since lipid bilayers in biological membranes are considered to be in a liquid-crystalline phase, the change of the membrane properties above the transition temperature has to be important from the physiological aspects. The increase of the bulk modulus due to the cholesterol incorporation is about 0.3×10^{10} dyn/cm^2, which is the same order of magnitude as the change in the bulk modulus due to the phase transition of pure DPPC membrane. That is, the membrane in the liquid-crystalline phase is much more stabilized against an external stress in the presence than in the absence of cholesterol.

In contrast to the dual effect on the bulk modulus, the density of the membrane decreases in both phases. When cholesterol is mixed into the membrane, the density decreases by about 6% in the gel phase and is only slightly changed in the liquid-crystalline phase. This fact appears to indicate that cholesterol enlarges the vacancy in the membrane, particularly in the gel phase. Since cholesterol is shorter than the hydrocarbon chain in DPPC by about four carbons, the incorporation of cholesterol has to give rise to vacancy near the methyl end of hydrocarbon chains (Shah & Schulman, 1967; Godici & Landsberger, 1975). Measurements of the mobility of each carbon in a chain by NMR (Darke et al., 1972) and ESR (Hubbel & McConnell, 1971) have verified the enhanced mobility near the nonpolar end. Therefore, it seems reasonable to assume that four carbons from the terminal methyl group are free from the steric effect of cholesterol. Then, by assumption of a 1:1 mixture of cholesterol and DPPC, enlarged vacancy is roughly calculated to be 8% of the total volume, which is in good agreement with the change in the density in the gel phase.

Although the stabilizing effect of cholesterol is apparent in the case of DPPC membrane, we must be careful in gener-

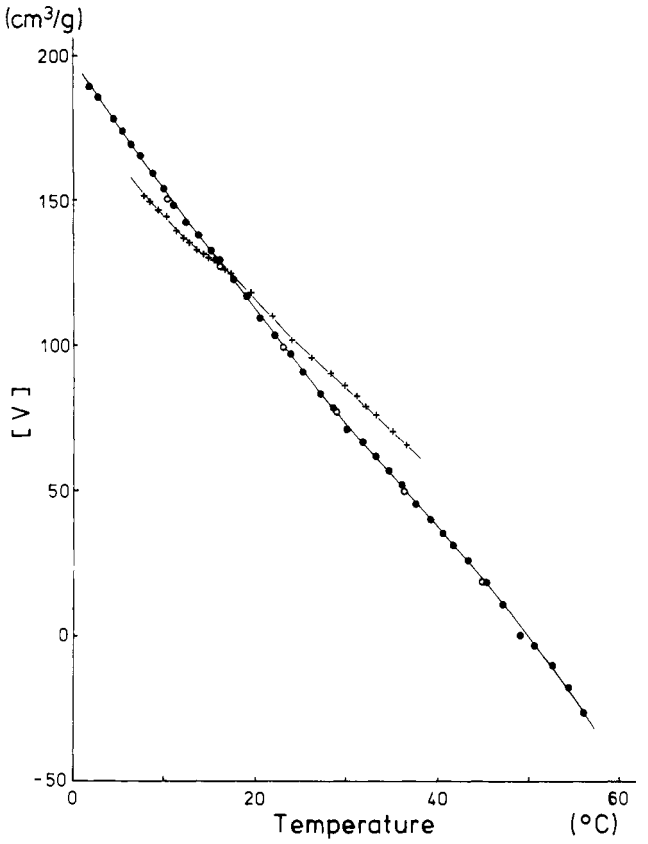

FIGURE 6: Temperature dependence of the limiting velocity number of human erythrocyte ghost suspension (+) (Sakanishi et al., 1976) and sonicated dipalmitoylphosphatidylcholine vesicles with 49.0% cholesterol (●, ○).

alizing this concept to biological membranes. Biological membranes contain many kinds of lipids and proteins and may be different from homogeneous systems like DPPC membranes. Therefore, we compare the results for DPPC–cholesterol membrane with membranes of biological origin: lipid vesicles of bovine brain sphingomyelin and human erythrocyte ghost.

Sphingomyelin from bovine brain contains hydrocarbon chains with heterogeneous chain lengths, which is a characteristic feature of biological membranes (Shinitzky & Barenholz, 1974). Barenholz et al. (1976) have reported that sphingomyelin from bovine brain shows a broad phase transition in the temperature range between about 25 and 45 °C, whereas synthetic sphingomyelin with monodisperse chain length undergoes a sharp transition at 41.3 °C. A polydispersity of chain length probably disturbs cooperative phase transition. The temperature dependence of the ultrasonic velocity in bovine brain sphingomyelin shows only a slight change between 34 and 43 °C, which is consistent with the results of Barenholz et al. (1976). Moreover, the curve of the temperature dependence falls between those of DPPC vesicles with cholesterol of 14.3 and 32.3%. Taking the work of Barenholz et al. (1976) into account, it appears that a critical fluctuation is restricted by the polydispersity of chain length, as cholesterol affects the properties of a membrane in the vicinity of the transition temperature. Therefore, it may be concluded that in biological membranes the long-range cooperativity of hydrocarbon ordering is partly restricted by the polydispersity of chain length and also suppressed by the presence of cholesterol.

Figure 6 shows the temperature dependence of the limiting velocity number of human erythrocyte ghost suspension (Sakanishi et al., 1976) together with the sonicated vesicles of DPPC containing cholesterol of 49.0%. The limiting velocity numbers of these membranes are almost equal. Since erythrocyte membranes contain approximately equimolar phospholipid and cholesterol, similar values of the limiting velocity number suggest that cholesterol stabilizes the erythrocyte membrane as it does in the artificial lipid membranes. The erythrocyte membrane is subject to high stress under physiological conditions and stabilization by cholesterol appears to be suited to the conditions. The temperature coefficient of the ultrasonic velocity in the erythrocyte membrane is a little smaller than that of DPPC–cholesterol membrane, and the two curves cross at about 20 °C. This difference may be due to the presence of proteins in erythrocyte membranes. However, more detailed study is necessary to discuss the structure of proteins in the membrane.

We have measured the ultrasonic velocity and the density of sonicated lipid vesicles and found that cholesterol stabilizes membranes. Critical fluctuation of hydrocarbon-chain packing is suppressed by the incorporation of cholesterol. Also, the bulk modulus of the membrane increases by about 15% when equimolar cholesterol is incorporated into the membrane above the transition temperature. From the comparison with erythrocyte membranes, it may be inferred that the role of cholesterol is to make a membrane harder as a barrier and that the membrane which has to be stable contains much cholesterol.

Acknowledgments

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Outer Doublet Tubulin Reassembly: Evidence for Opposite End Assembly–Disassembly at Steady State and a Disassembly End Equilibrium†

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ABSTRACT: [^{3}H]GTP exchangeably bound to outer doublet tubulin becomes nonexchangeable with exogenous GTP upon incorporation of the tubulin into microtubules in vitro. We have used this property to study the mechanism of outer doublet tubulin exchange with reassembled microtubules in vitro. At apparent equilibrium, net addition and loss of tubulin occur at opposite ends of the microtubules. The apparent equilibrium is actually a steady-state summation of two different reactions which occur at the opposite microtubule ends, which results in a unidirectional flux of tubulin from microtubule assembly ends to disassembly ends. The similarity of this behavior with that shown previously for bovine brain microtubules in vitro [Margolis, R. L., & Wilson, L. (1978) *Cell 13*, 1] supports our earlier contention that neither the doublet structure nor the stability of outer doublet microtubules in situ is determined solely by the tubulin backbone. Further, the intrinsic assembly–disassembly behavior of tubulins from very diverse sources is strongly conserved. Tubulin loss is readily reversible at the disassembly ends of microtubules polymerized from outer doublet tubulin, indicating that these ends are in equilibrium with tubulin in solution. This does not appear to be the case with bovine brain microtubules that have been assembled in vitro. In addition, the kinetics of podophyllotoxin-induced microtubule depolymerization suggest that addition of podophyllotoxin–tubulin complexes may occur at the microtubule diassembly ends under non-steady state conditions. Thus despite the strong conservation of assembly properties of tubulin from stable sea urchin sperm tail outer doublet microtubules and bovine brain, some differences do exist. These differences may reflect differences in the cellular functions of microtubules.

The in vitro reassembly of tubulin from stable outer doublet microtubules is very similar to that of tubulin from vertebrate brain. In addition, the characteristics of the reassembled microtubules closely resemble those of labile brain microtubules, rather than the stable outer doublet microtubules from which the tubulin was derived (Kuriyama, 1976; Farrell & Wilson, 1978; Binder & Rosenbaum, 1978; Farrell et al., 1979). These results indicate that the polymerization properties of tubulins have been highly conserved and suggest that neither doublet formation nor the stability of microtubules is determined solely by the tubulin subunits (Farrell et al., 1979).

It is possible that other proteins associated with microtubules determine the stability and functions of the microtubules, while chemical heterogeneity among tubulins (Witman et al., 1972; Bibring et al., 1976; Feit et al., 1977; Kobayashi & Mohri, 1977; Marotta et al., 1978; Stephens, 1978) could provide the basis for functional specificity in assembled microtubules.

We have continued our studies on the control of microtubule properties and function by examining the mechanism of tubulin dimer exchange with microtubules reassembled in vitro from outer doublet tubulin. It has been shown previously that tubulin dimer addition and loss occur at opposite ends of microtubules polymerized in vitro from bovine brain tubulin at steady state (Margolis & Wilson, 1978; Wilson & Margolis, 1978). The ability of PLN[1] to poison reassembly of outer doublet tubulin substoichiometrically (Farrell et al., 1979) indicated that a similar assembly–disassembly mechanism existed for microtubules reassembled from outer doublet tubulin.

Determination of the mechanism of tubulin dimer exchange in microtubules reassembled from outer doublet tubulin is of interest for two reasons. First, outer doublet tubulin is derived from microtubules that differ in morphology (Warner, 1972), stability (Behnke & Forer, 1967), and tubulin chemistry (Bibring et al., 1976; Stephens, 1978) from cytoplasmic microtubules such as those derived from vertebrate brain. Although the previous studies have indicated that a number of microtubule properties are not determined solely by the tubulin, a more detailed analysis of the mechanism of tubulin dimer exchange may reveal subtle differences between outer doublet and cytoplasmic tubulins which are functionally important in the assembled microtubules. Secondly, microtubules reassembled in vitro from outer doublet tubulin and

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[1] Abbreviations used: Mes, 2-(*N*-morpholino)ethanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*′-tetraacetic acid; PLN, podophyllotoxin; CLC, colchicine; MAPs, high molecular weight microtubule associated proteins.

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