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Effect of Saccharides on the Freezing and Thawing of Liposome Dispersion

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The cryoprotective activities of saccharides on liposome dispersion prepared from a mixture of egg yolk lecithin and dicetylphosphate (10:1 in molar ratio) were investigated in terms of the leakage of the entrapped marker (calcein), and the changes of the turbidity and the half band-width of CH_2 signals in the ^{1}H-NMR spectrum. In the presence of more than 100 mM D-glucose in both the inner and outer aqueous phases of liposomes (concentration of phospholipid; 6.4 mM), the liposome dispersion was stable to freezing at -70 °C. D-Galactose, D-mannose, maltose and maltotriose showed similar activities to D-glucose. However, maltopentaose and DextranT-100 had no cryoprotective effect on the liposome dispersion. Sodium chloride, potassium chloride and urea also showed cryoprotective activities above their eutectic temperatures. The mechanism of the cryoprotective action of saccharides on the freezing of liposomes is discussed in terms of differential scanning calorimetry data and Raman spectra of aqueous saccharide solutions at low temperature.

Keywords——liposome; freezing–thawing; differential scanning calorimetry; Raman spectra; unfreezable water; cryoprotection; leakage; turbidity; calcein-fluorometry; eutectic temperature

Various saccharides, dimethylsulfoxide and polyalcohols such as glycerin are known to act as cryoprotective agents for living cells.[1] These compounds also prevent protein denaturation during freezing. The mechanism of the cryoprotection may involve protection of membrane from mechanical damage by ice crystals and the prevention of conformational change of membrane protein (denaturation) by the concentrated salt solution produced during the freezing process. The cryoprotective activities of monosaccharides for water-soluble proteins may be based on the formation of hydrogen bonding between the protein molecule and saccharide molecules instead of water molecules.[2] Monosaccharides protect protein in aqueous solution from denaturation from heating. However, little work has been done on the interaction of monosaccharides with lipid membrane.

Recently lipid vesicles constructed from bilayers of phospholipid molecules, so-called "liposome" have been extensively studied as a model membrane and a potential drug carrier in the pharmaceutical field. One of the most important problems for the practical use of liposomes as a drug delivery system is the low stability, since leakage, fusion and phase separation may occur on storage of the aqueous dispersion. Freezing,[3-5] and freeze-drying[4-6] have been attempted with addition of various compounds to the outer aqueous

phase of the liposome dispersion to improve the stability of liposomes and some success was achieved with lactose in the outer aqueous phase.[4] However, the mechanism is still an open question. At temperatures below the freezing point, the lipid bilayer is usually in a gel state, where the permeability to water-soluble compounds may be low. Therefore freezing is a promising approach for the preservation of liposomes if the lipid bilayer remains intact in the frozen state.

Thus, we have investigated the cryoprotective activities of various solutes during the freezing–thawing process of liposome dispersion in terms of the leakage of marker compound, the turbidity of the dispersion and the change of the proton nuclear magnetic resonance (^{1}H-NMR) spectrum. Initially, we investigated the frozen states of aqueous solutions of additives, especially urea and α-D-glucose in order to elucidate the mechanism of cryoprotection.

Experimental

Materials——Saccharides (D-glucose, D-galactose, D-mannose, maltose, maltotriose and maltopentaose) were obtained from Nakarai Chemical Co., Ltd., and were used after drying *in vacuo* for 7 d at room temperature. Dextran T-100 and Sephadex G-50 were products of Pharmacia Co., Ltd., and were used without further purification. Reagent-grade urea, sodium chloride, potassium chloride, mannitol, cobalt chloride and Mops (3-(*N*-morpholine)propanesulfonic acid) were obtained from Nakarai Chemical Co., Ltd., and were used after drying *in vacuo* at room temperature. Calcein (2,4-bis(*N*,*N*-di(carboxymethyl)aminoethyl) fluorescein) was obtained from Tokyo Kasei Co., Ltd., and was used without further purification. Egg yolk lecithin and dicetylphosphate were purchased from Sigma Chemical Co., Ltd., and were used without further purification. Reagent-grade D_2O and NaOD were obtained from Merck Co., Ltd. Water was purified by distillation and deionization.

Methods——Differential scanning calorimetry (DSC) thermograms were obtained by using Shimadzu DSC-30 apparatus. Various concentrations of aqueous sample solutions were weighed in aluminum sealing pans and the DSC thermograms were obtained at a heating rate of 5 °C/min under a helium atmosphere after cooling to −70 °C with liquid nitrogen (cooling rate, about 10 °C/min). Measurement was done at least twice for each concentration and the heat absorbed was determined from the area under the peaks using the heat of melting of ice as a standard.

NMR spectra of liposomes in D_2O were measured by JEOL FT-NMR-200 at room temperature. The leakage of entrapped calcein was determined by measurement of the fluorescence intensity at 520 nm with excitation at 490 nm using a Jasco FB-550 fluorometer. The turbidity of liposome dispersion was determined by measuring the absorbance at 600 nm with a double-beam spectrophotometer (Shimadzu Co., Ltd.). Raman spectra of aqueous solutions of urea and D-glucose were measured with a JEOL S-1 laser Raman spectrophotometer with a cryostate cell for low temperature measurements.

Preparation of Liposomes——Take 0.9 ml (concentration: 40 mg/ml) and 0.6 ml (concentration: 4 mg/ml) of chloroform solutions of egg yolk lecithin and dicetylphosphate (DCP), respectively, in a pear-shaped flask, and evaporate the mixed solution to dryness in a rotary evaporator. The thin film thus obtained was further dried *in vacuo* for 3 h at room temperature and then mixed with 2.5 ml of 10 mM Mops buffer solution containing 4.0×10^{-4} M calcein and various concentrations of additives. The mixture was vortexed in hot water (30—40 °C) for several minutes and then sonicated at 0 °C under a nitrogen atmosphere for 30 min with a probe-type sonicator (model UR 200P) manufactured by Tomy Seiko Co., Ltd. The vecicle dispersion thus obtained was filtered through a Millipore filter (pore size: 3 μm) to remove titanium oxide. It was confirmed by electonmicroscopy that the liposome dispersion consisted of uni- and oligolamellar vesicles with an average diameter of 55 ± 5 nm. The initial concentration of egg yolk lecithin and DCP were 20 and 2 mM, respectively. Gel chromatography was employed to remove the calcein in the outer aqueous phase with 10 mM Mops buffer (pH 7.2) and additives as the eluent. The final concentration of phospholipid in the dispersion was 6.4 mM.

Freezing and Thawing Procedure——Freezing of the vesicle dispersion was carried out by immersing the test tube containing the liposome dispersion directly (fast cooling: 100 °C/min) or indirectly (slow cooling: 5 °C/min) in a cooling agent (usually dry ice–methanol). For freezing at constant temperature, we used the thermo-regulated chamber of a freeze-drying apparatus; the temperature was determined from the electric resistance of a thermocouple immersed in the liposome dispersion. The frozen solid was usually kept for 2 h at each temperature and then thawed at room temperature. The measurements of ^{1}H-NMR spectrum, fluorescence and turbidity were carried out 1 h after melting of the ice.

Calculation of Percent Leakage of Calcein——The fluorescence intensity (F) is defined as the peak height of the emission spectrum at 520 nm with excitation at 490 nm. The percent leakage is calculated from Eq. 1.

$$\text{leakage } (\%) = \frac{(F_1 - F_2) - (F'_1 - F'_2)}{F_1 - (F_3 - F_4)} \times 100 \qquad (1)$$

where F_2 and F_1 are the fluorescence intensities of calcein entrapped in liposomes after freezing and thawing with or without addition of cobalt chloride[7] in the outer aqueous phase, respectively, and F_2' and F_1' are the fluorescence intensities of the original liposomes with or without addition of cobalt chloride in the outer aqueous phase, respectively.[8] F_3 is the fluorescence intensity of calcein entrapped in liposome after addition of Triton X-100 and cobalt chloride consecutively.[9] F_4 is the fluorescence intensity of calcein-free liposomes after freezing and thawing.[10]

Results and Discussion

The DSC thermograms of various concentrations of D-glucose solutions at a heating rate of 5 °C/min and the phase diagram are shown in Figs. 1 and 2, respectively. As shown in Fig. 1, aqueous solutions of D-glucose show only a peak due to the melting of ice crystals and do not show an endothermic peak due to the melting of the eutectic mixture, in spite of a report that aqueous D-glucose solution has a eutectic point at 268 K, and the mole fraction of D-glucose is 0.32.[11] Aqueous solutions of D-galactose, D-mannose, maltose, maltotriose and maltopentaose showed similar behavior. Several papers have reported the presence of unfreezable water in aqueous solutions of saccharides below the freezing point.[11-13]

As shown in Fig. 3, the heat of melting per gram of aqueous D-glucose solution (H_f/g) is linearly related to the weight fraction of D-glucose. From the intercept at $H_f = 0$, we calculated the amount of unfreezable water. Table I shows the numbers of unfreezable water molecules

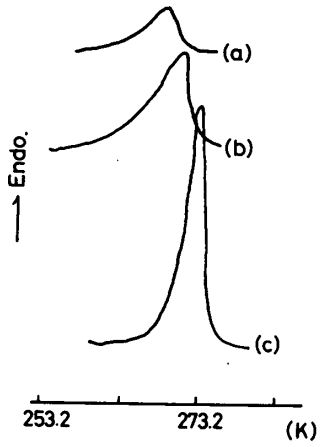

Fig. 1. DSC Thermograms of the D-Glucose–Water System

Weight fraction of D-glucose: (a) 0.420, (b) 0.326, (c) 0.163.

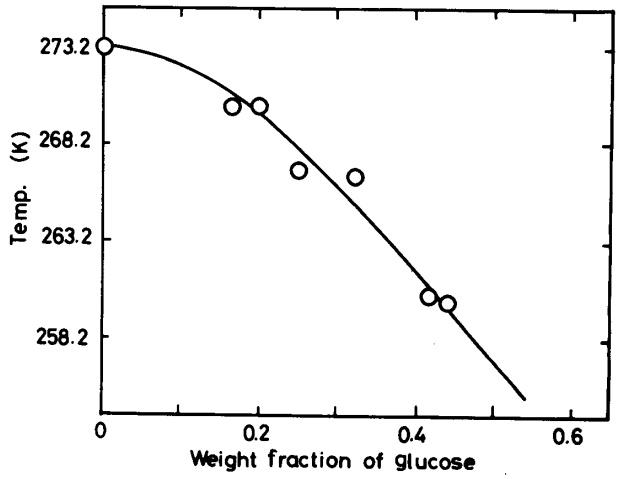

Fig. 2. Phase Diagram of the D-Glucose–Water System

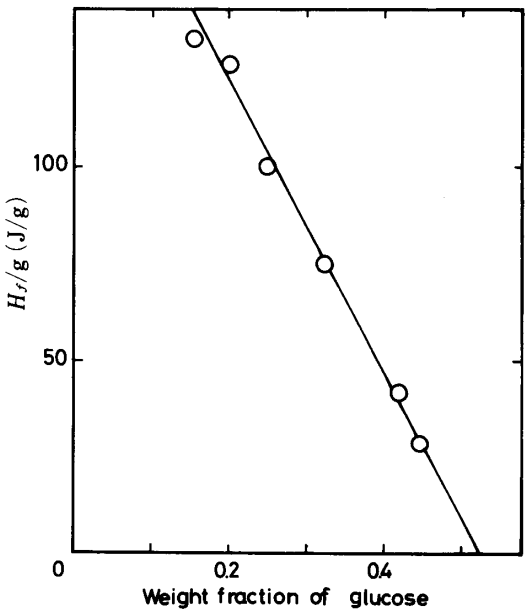

Fig. 3. Heat of Fusion of Ice *vs.* Weight Fraction of D-Glucose

TABLE I. Unfreezable Water for Various Saccharides

Saccharide	n	N	N/n
D-Glucose	1	9.5	9.5
D-Galactose	1	9.2	9.2
D-Mannose	1	8.2	8.2
Maltose	2	19.5	9.8
Maltotriose	3	23	7.7
Maltopentaose	5	36	7.2

n: numbers of monosaccharide units. N: numbers of unfreezable water molecules per saccharide molecule.

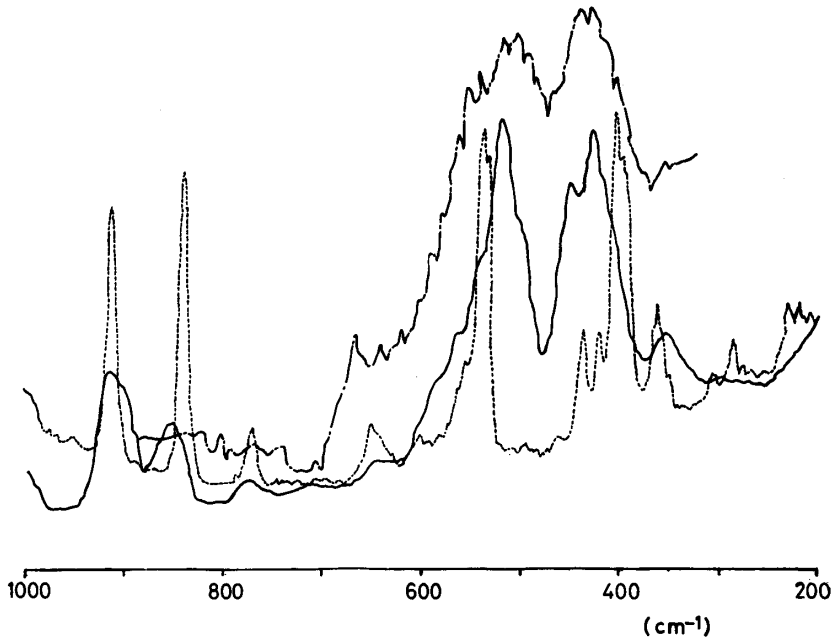

Fig. 4. Raman Spectra of D-Glucose

------, crystals of α-D-glucose; ——, 50% (w/w) aqueous D-glucose solution at 25 °C; – – – –, frozen solid of 50% (w/w) aqueous D-glucose solution at −40 °C.

per saccharide molecule for various saccharides. The number of unfreezable water molecules per glucose residue tends to decrease with increase of glucoside linkages. The numbers of unfreezable water molecule among the three epimers are not very different from each other. A similar experiment was done with a dispersion of lecithin and a value of 10 molecules of water per lecithin molecule as unfreezable water, was obtained.

In contrast with aqueous D-glucose solution, aqueous urea solution showed two endothermic peaks on heating of the frozen mixture. The eutectic temperature and composition were 258.4 K and 0.242 (weight fraction of urea), respectively.

In order to elucidate the state of D-glucose molecules at low temperature when all the freezable water has crystallized, we measured the Raman spectra of aqueous D-glucose solution (50%(w/w)) at 25 and −40 °C, together with that in the solid state of α-D-glucose. The sample solution was cooled by using a cryostat cell at a cooling rate of 10 °C/min. These Raman spectra are shown in Fig. 4. For comparison, a similar experiment was carried out with urea. The spectrum of urea crystals coincided with that of frozen solid eutectic mixture at −40 °C, and was different from that of the eutectic composition at 25 °C, indicating that the urea molecules in the frozen solid are in the crystalline state. In contrast to urea, the Raman spectrum of D-glucose solution at −40 °C resembles that of D-glucose at 25 °C. The bands at

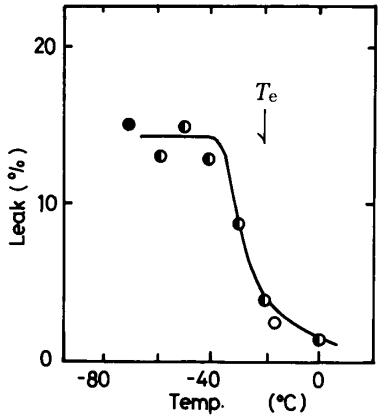

Fig. 5. Percent Leakage as a Function of Freezing Temperature for Liposomes Containing 100 mM NaCl

◑, slow cooling; ●, rapid cooling; ○, slow cooling with the mixture of ice and ammonium sulfate.

TABLE II. Percent Leakage and Change of Turbidity (ΔOD_{600}) after Freezing and Thawing in the Presence of Various Additives

Additive	T_e (°C)	% leakage		ΔOD_{600}	
		$T>T_e$	$T<T_e$	$T>T_e$	$T<T_e$
Sodium chloride	−21.1	2.5	5.7	0.02	0.28
Potassium chloride	−10.7	2.0	15.5	n.d.	
Urea	−15.0	1.0	23.0	0.02	0.19
Mannitol	−2.5	—	35.0	—	0.34
None	—	—	51.0	—	0.70

T: freezing temperature. T_e: eutectic temperature. n.d., not determined.

424 and 450 cm^{-1} in aqueous solution at 25 °C are the bending vibration of C–C–O and C–C–C, respectively. The bands observed at 518 and 540 cm^{-1} in aqueous solution at 25 °C are bending vibrations of C2–C1–O1 of α-D-glucose and β-D-glucose, respectively.[14] In aqueous solution of D-glucose at equilibrium, the anomeric ratio of α- to β-form is about half. The Raman spectrum of aqueous D-glucose solution reflects this situation. The Raman spectrum of the frozen solid of α-D-glucose solution at −40 °C obtained by slow cooling is similar to that of aqueous solution at 25 °C with respect to the band positions and shape, indicating that D-glucose molecules are not in a crystalline state, but in a liquid or glassy state. Glass transition of 60% (w/w) aqueous solution of D-glucose takes place below −80 °C. Therefore, a concentrated aqueous solution of D-glucose should exist in the interstices of ice crystals at −40 °C.

Effect of Additives on the Leakage of Calcein during the Freezing and Thawing Process

The effect of freezing temperature on the leakage of calcein in 100 mM NaCl solution is shown in Fig. 5. The open circle in Fig. 5 is the point obtained from the experiment with a mixture of ice and ammonium sulfate as a cooling agent. The percent leakage increases abruptly at the eutectic temperature (−21.5 °C). The cooling rate hardly affects the percent leakage between 5 °C/min and 100 °C/min. The effect of various additives on the percent leakage after freezing below and above the eutectic temperature (T_e) are shown in Table II. The difference in the percent leakage below and above T_e is significant, indicating that mechanical breaking of the membrane arising from crystal formations of ice and additive play an important role in the leakage of calcein, as found in the microscopic study by Siminovitch and Chapman.[15] Since the gel–liquid crystal transition temperature (T_c) of egg yolk lecithin is between −2 and −15 °C, the lipid bilayer should be in a gel state. The highest percent leakage was observed in the liposome dispersion without an additive, suggesting that the large ice

TABLE III. Percent Leakage and ΔOD_{600} after Freezing and Thawing in the Presence of Monosaccharides

Monosaccharide	% leakage	ΔOD_{600}
D-Glucose	4—7	0.01—0.02
D-Galactose	3—4	0.01
D-Mannose	4—5	0.01
None	51.0	0.70

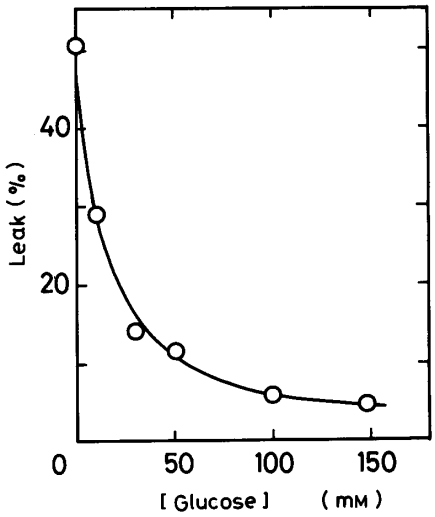

Fig. 6. Percent Leakage of Calcein as a Function of the Concentration of D-Glucose

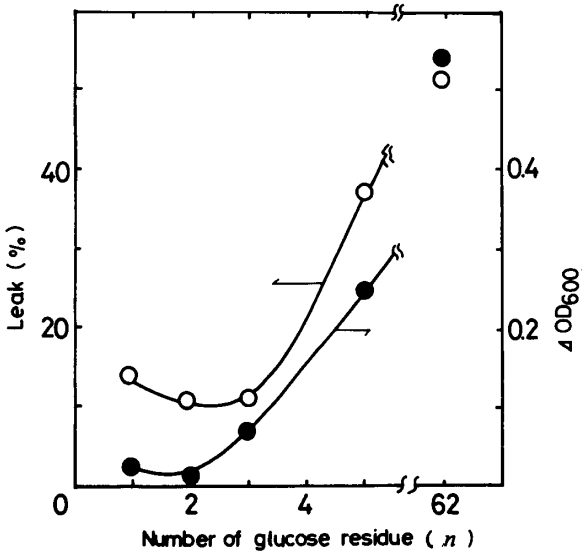

Fig. 7. Percent Leakage as a Function of the Numbers of Glucose Residues

●, ΔOD_{600}; ○, percent leakage (concentration of saccharide was 30 mM except Dextran T-100, 10 mM).

crystals formed in the frozen state effectively destroy the structure of the bilayer membrane. The relatively low leakage in the presence of NaCl and KCl may be due to the promotion of fusion and reconstruction of liposomes at the initial stage of melting of the eutectic mixture, because these salts enchance hydrophobic interaction in aqueous solution.

Effect of Additives on the Turbidity of Liposome Dispersion

The ΔOD_{600} is defined as the difference of absorbance at 600 nm before and after freezing and thawing. Therefore, large values of ΔOD_{600} indicate that liposome destruction and fusion take place during the freezing and thawing process. As can be seen in Table II, the values increase drastically when the dispersions are cooled below T_e of each additive. This result is consistent with the results of the leakage experiment described above.

Effect of Saccharides on the Percent Leakage and ΔOD_{600}

Table III shows the effects of monosaccharides on the leakage of calcein and the value of ΔOD_{600} in the freezing and thawing process. In contrast to the other additives cooled below their eutectic temperature, monosaccharides protect liposomes from destruction and fusion on freezing to $-70\,°C$. Both the percent leakage and the values of ΔOD_{600} are quite small. Judging from the fact that the percent leakage and the values of ΔOD_{600} are also small when the liposome dispersion with an additive forming a eutectic mixture is cooled above T_e, the presence of a concentrated aqueous layer, that is, a fluid layer in close vicinity to the bilayer membrane seems to be an essential factor for the stability of liposomes below the freezing

point. As shown in Fig. 6, the minimum concentration required for protection from freezing damage is about 100 mM D-glucose per 6.4 mM liposome dispersion.

As shown in Fig. 7, the oligomers of D-glucose have different effects on the stability of liposome dispersion upon freezing. D-Glucose, maltose and maltotriose stabilize the liposomes, while maltopentaose showed little effect. Furthermore, Dextran T-100 is a destabilizer in spite of the large amount of unfreezable water. As can be seen in Table I, saccharide molecules generally have unfreezable water, which is bound tightly through hydrogen bonding. This unfreezable water is presumably involved in the unfrozen layer around the liposome surface. However, as seen in Fig. 7, maltopentaose, which has large amount of unfreezable water, shows weak cryoprotective activity.

In general, the saccharide molecule has a weak hydrophobic character, which originates from the hydrophobic surface formed by the CH- and CH_2-groups of the saccharide molecule.[16] The hydrophobicity increases with number of glucose residues.[17] Cyclodextrins are the extreme cases; they form inclusion complexes with phospholipid molecules.[18] Therefore, these oligomers and polymer may interact with membrane phospholipid molecules through hydrophobic bonding and pull the phospholipid molecules out of the bilayer, resulting in destruction of the bilayer structure and leakage of the encapsulated marker.

Effect of Freezing of Liposomes on the ^{1}H-NMR

To examine the change of membrane structure caused by freezing, the ^{1}H-NMR spectra of liposome in D_2O solution containing various additives were measured before and after freezing and thawing. As shown in Fig. 8, the half band-width of the methylene proton signal from the acyl chain of egg yolk lecithin without additive increases after freezing and thawing, indicating that the mutual interaction of neighboring acyl chains is stronger, that is, the formation of large vesicles or a flat lamellar phase takes place.

On the other hand, the half band-width of the methylene proton signal due to the acyl chain of lecithin in the presence of D-glucose is almost unchanged before and after freezing and thawing, indicating that the original liposomes were not destroyed in this process. The addition of 100 mM D-glucose to only the outer aqueous phase of liposome dispersion also prevents the leakage of entrapped calcein during freezing. The percent leakage was about

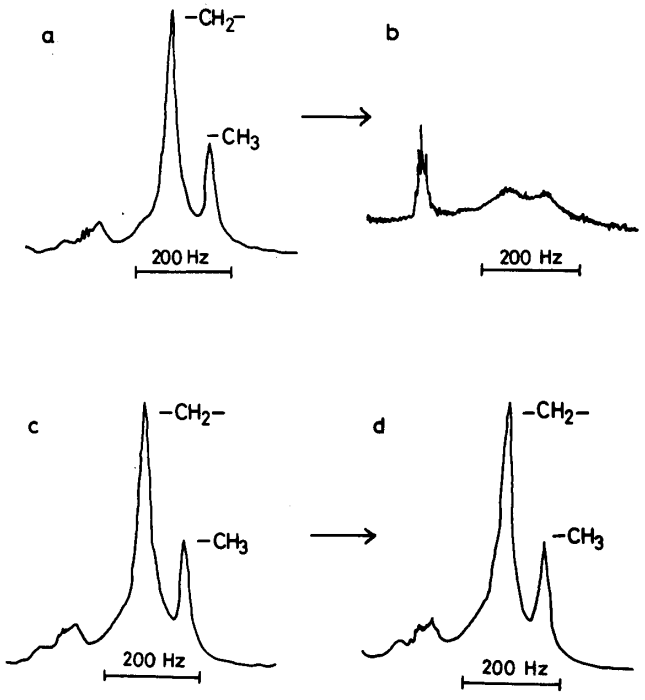

Fig. 8. ^{1}H-NMR Spectra of CH_2- and CH_3-Groups of Liposome Dispersion

a: Liposomes containing aqueous solution of buffer before freezing.
b: Liposomes containing aqueous solution of buffer after freezing and thawing.
c: Liposomes containing aqueous solution of buffer and 100 mM D-glucose before freezing.
d: Liposomes containing aqueous solution of buffer and 100 mM D-glucose after freezing and thawing.

TABLE IV. Half Band-Width of the Methylene Proton Signal before and after Freezing and Thawing in the Presence of Various Additives

		Additive			
		D-Glucose	NaCl	Mannitol	No additive
Half band-width (Hz)	Before freezing	34.3	40.0	34.2	34.2
	After freezing and thawing	37.1	45.7	68.6	Not measurable

Concentration of additive: 100 mM.

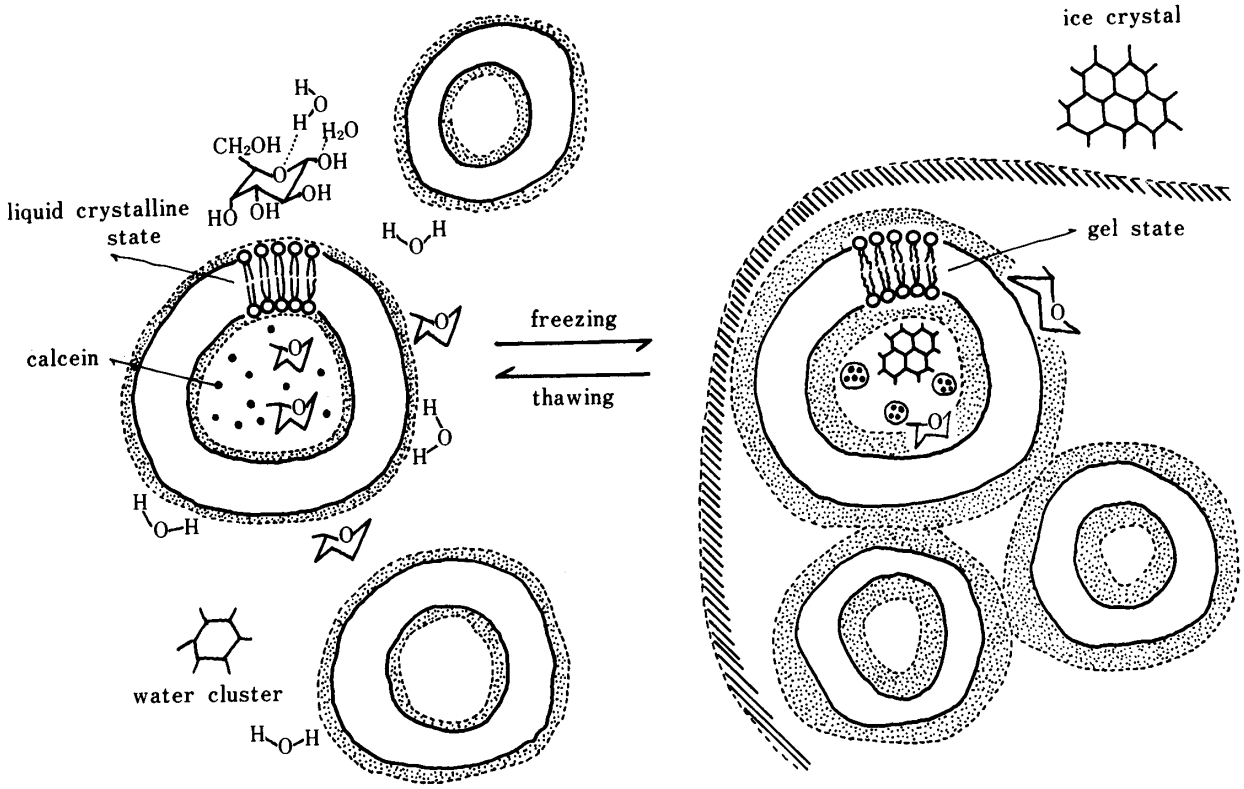

Fig. 9. Schematic Representation of Freezing and Thawing of Liposome Dispersion Containing D-Glucose and Solute

12%, which is about twice that of liposomes containing the same concentration of D-glucose in both the inner and outer aqueous phase. This may be due to the osmotic difference and the destruction of the bilayer membrane by the ice crystals grown in the inner aqueous phase.

The effects of various additives on the half band-width of the methylene proton signal are shown in Table IV. The degree of widening of the half band-width for each additive is consistent with the leakage and turbidity data shown in Table II.

Based on the above discussion, the mechanism of cryoprotection of the saccharide is proposed to be as illustrated in Fig. 9. At room temperature, liposomes whose bilayer is in the liquid crystal state, are dispersed in aqueous D-glucose solution. The hydration layer may be thin. In the process of freezing, the liposomes and D-glucose in the aqueous phase are concentrated between the interstices of growing ice crystals, and the surface of the liposomes is covered with a concentrated aqueous D-glucose layer, where the ice crystals cannot penetrate. Now the lecithin bilayer is in a gel state with low permeability to the entrapped calcein. The concentrated aqueous D-glucose layer would also prevent the aggregation and fusion of liposomes. The reverse process will take place during the thawing of liposome

dispersion containing D-glucose. The other additives below their eutectic temperature form eutectic mixtures, which break the bilayer membrane mechanically.

In conclusion, the freezing of liposomes containing saccharide in both aqueous phase seems to be a promising approach for the preservation of liposome dispersions.

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8) The term $(F_1' - F_2')$ is usually quite small or nearly zero.
9) In the presence of liposomes, the fluorescence of calcein is not completely quenched by the addition of excess cobalt chloride.
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