  Bull. Chem. Soc. Jpn., 64, 1588—1593 (1991)

Characteristics of the Membrane Permeability of Temperature-Sensitive Liposome

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(Received December 20, 1990)

The relationship between the temperature-sensitivity of the release of an entrapped drug from liposome as well as the type of drug or liposome is discussed. The drug release from DPPC liposome (dipalmitoylphosphatidylcholine liposome) has been confirmed to increase abruptly near its phase-transition temperature for both low-molecular-weight compouds (such as calcein) and high-molecular-weight compounds (such as dextran). The amount of temperature-sensitive calcein released from the liposomes was dependent on the method of liposome preparation: that is, the release from REV (reverse phase evaporated vesicle) was the highest; next was that from LUV (large unilamellar vesicle prepered by detergent removal method), and lowest was from SUV (small unilamellar vesicle prepared by an ultrasonication method). In order to examine the size effect of liposomes on temperature-sensitive drug release, REV was fractionated into three groups according to their average sizes and calcein release for each group was investigated. The temperature at which calcein release showed a maximum was almost the same in each of three groups; while as the average liposome size of a group was larger, the initial rate of calcein release became higher and the temperature at which calcein release began became lower. The amount of release of a entrapped drug was primarily dependent on the molecular weight of the drug, in spite of the variety of drugs investigated.

Liposomes have been regarded as having promise for use as a new type of drug carrier. They can encapsulate either hydrophilic or hydrophobic drugs in their inner core or membrane matrix. They can be expected to have different forms of interactions with bioorgan than do other types of drug carriers, such as emulsions or microspheres, judged from a similarity with biological cell membranes regarding both structure and nature. They can also be considered as being biodegradable and to have little toxity.[1,2] One obstacle in putting liposomes to practical use as drug carriers has been a difficulty in controlling the release of encapsulated drugs. Yatbin et al.[3] and Weinstein et al.[4] showed the possibility of medical applications of the so-called "temperature-sensitive liposome", which enables the control of drug release from a liposome using the nature of the phospholipid membrane; the barrier efficiency of the membrane abruptly decreases near the phase transition temperature of a gel-to-liquid crystalline of the phospholipid membrane.[5] This report concerns an extension of their idea. In order to extend the application of a temperature-sensitive liposome, it is especially important to clarify the characteristics of the temperature-sensitive membrane permeability. In this report the effects of various entrapped drugs and the forms of liposomes on the temperature-sensitive drug release were studied. Especially, results concerning the possibility of controlling the release of macromolecule drugs would stimulate the use of temperature-sensitive liposomes for medical applications, since many macromolecular drugs, such as urokinase, are easily inactivated by a variety of inhibitors in the blood;[6] also, their clearance from blood is very rapid.

Experimental

Materials. L-α-phosphatidylcholine from egg yolk (egg PC) was purchased from Green Cross Co. (no less than 99% purity). L-α-dipalmitoylphosphatidylcholine (DPPC) was obtained from Sigma Co. (no less than 99% purity determined by thin-layer chromatography). *N*-octyl β-D-glucoside and calcein (3,3′-bis[*N*,*N*-(dicalboxymethyl)aminomethyl]fluorescein) were obtained from Dojindo Laboratories. Dextran ($\overline{M}_W$; 70000) and inulin were purchased from Wako Chemicals. Urokinase was obtained from Kyorin Co, (the trade name is caltokinase). Sinthesized fluorescenced urokinase substrate, Glutaryl-Gly-Arg-MCA (4-methylcoumarinamide), was obtained from Protein Research Foundation. ^{3}H-dextran (specific activity; 336 μCi mg^{-1}) and ^{14}C-inulin (specific activity; 2.6 μCi mg^{-1}) were obtained from Amercham. Hydrophobic beads used for detergent removal were Amberlite XAD-2 obtained from Tokyo Organic Chemicals. All other reagents were of reagent grade from Wako Chemicals or Nakarai Chemicals.

Methods. Liposome Preparation. DPPC-SUV (small unilamellar vesicle) was prepared according to a method of Weinstein et al.[4] or Watt et al.[7] as follows: a dried, thin film of dipalmitoylphosphatidylcholine was swollen with buffer solution, (if necessary, containing an appropriate drug,) for 2 min at 50°C followed by shaking for 20 s; this process was repeated 3 times. The resulting suspension was sonicated using a probe-type sonicator (Taiyo Seiki Co., model UR-200P) at 50 °C for 60 min, followed by standing for 15 min at 50 °C. The suspension was then ultracentrifuged at 100000×*g* for 15 min using a centrifuge (Hitachi automatic preparative ultracentrifuge, 55p-72). SUV was obtained from the supernatant.

DPPC-LUV (large unilamellar vesicle) was prepared according to a method described in previous papers[8-10] as follows: a dried thin film of phospholipid was solubilized by octyl glucoside (molar ratio of pospholipid to octyl glucoside was 1/10);

the solubilized micellar solution was rapidly passed through a XAD-2-loaded column at 50 °C. The liposomes prepared by this method have been known to be mostly unilamellar vesicle. LUV was obtained after 15 passages through the column.

DPPC- or EggPC-REV (reverse-phase evaporated vesicle) was prepared according to a method of Szoka et al.[11] The preparation of an emulsion during this procedure was accomplished using diisopropyl ether / chloroform (1/1) at 47 °C for DPPC-REV preparation, and diethyl ether at 4 °C for EggPC-REV.

Any untrapped drug was removed by centrifugation for REV or gel chromatography using Sephadex G-50 for LUV or SUV. The drug-loaded liposomes were resuspended in Tris-HCl buffer (150 mM NaCl / 10 mM Tris-HCl / pH 7.4, 1 M=1 mol dm^{-3}) and, if necessary, concentrated or diluted to make an appropriate concentration for succeeding release experiments.

Size Fractionation of REV. Since REV contained various sizes of liposomes, it was fractionated by centrifugation as follows: First, REV suspension was centrifuged at 30000×*g* at 4 °C for 20 min. Small REV (S-REV) was obtained from the supernatant. The precipitate was resuspended in Tris-buffer after being washed by a cold buffer 3 times, and centrifuged at 1000×*g* at 4 °C for 20 min. Medium REV (M-REV) was obtained from the supernatant and large REV (L-REV) from the precipitate. These suspensions were either diluted or concentrated to make an appropriate concentration for the succeeding release experiment.

Entrapping Efficiency and Captured Volume. The entrapping efficiency and captured volume were evaluated by adding a fluorescence quencher in a calcein-containing liposome suspension[12] as follows: liposome was prepared in 10 μM of a calcein solution; the remaining fluorescence intensities were measured after the fluorescence of untrapped calcein was quenched by the addition of an appropriate amount of 100 mM $CoCl_2$. The entrapping efficiency was calculated by using the following equation:

$$\text{entrapping efficiency (\%)} = \frac{f_{in}\, r_1 - f_{total,q}\, r_2}{f_{total} - f_{total,q}\, r_2} \times 100,$$

where f_{in} is the fluorescence intensity of the internal calcein; f_{total} and $f_{total,q}$ are the total fluorescence intensity of the calcein before and after liposome destruction, respectively; r_1 and r_2 are correction factors for the volume increase upon adding a $CoCl_2$ solution and the Triton X-100 solution, respectively. They were 2620/2600 and 2625/2600 in our experiments. The fluorescence intensity of calcein was measured by a fluorophotometer (Shimadzu, difference spectrofluorophotometer, RF-503A) at 490 nm for exitation and 520 nm for emission. The captured volume was defined as being the total internal volume per mole of phospholipid, calculated from the amount of drug captured.

Release Measurement of Entrapped Drug. Calcein release was evaluated by a fluorescence self-quenching method according to Yatvin et al.[3] or Senior et al.[13]; 20 μl of a liposome suspension containing 100 mM of calcein was added to 2.5 ml of a buffer solution which had been kept at the desired temperature in advance. With this method the temperature was rapidly controlled. ^{14}C-inulin and ^{3}H-dextran release was monitored by measuring their radioactivity (using an Aloka scintilation system, LSC-900) in the supernatant by centrifugation at 40000×*g* for 20 min at 4 °C after an appropriate incubation time of the liposome suspensions at the desired temperature.

Urokinase release was monitored enzymatically using a fluorescenced urokinase substrate according to the method of Morita et al.[14]

Other Methods. The liposome size was evaluated by quasielastic light scattering (QELS) using a laser particle analyzing system (Otsuka Electronics Co. LPA 3000/3100). The phase-transition temperature was monitored by calorimetry using a differential scanning calorimeter (2nd Seiko Co. SSC-560). Phospholipid concentrations were determined by the micro method of Bartlet.[15]

Results

Characterization of Liposomes. The size and entrapping efficiency of liposomes prepared by several methods are summarized in Table 1. REV was the largest regarding both size and entrapping efficiency. The stability or barrier efficiency of DPPC-REV in a buffer solution at 4 °C are shown in Table 2. About 90% of the low-molecular-weight compound (calcein) and about 100% of the high-molecular-weight compound (dextran) were retained in the liposomes after 4 weeks of incubation. Judged from these observations, the liposomes could be regarded as having a high barrier efficiency against both low- or high-molecular-weight compounds at a temperature below their phase-transition temperature.

Calcein Release and Phase Transition. In order to examine the characteristics of the temperature-sensitive membrane permeability, the release of calcein from the liposome was investigated under several conditions. In Fig. 1, DSC curve of DPPC-REV and calcein release (%) from the liposome after 2 min incubation at several temperatures are shown. An endthermal peak due to the phase transition of DPPC hydrocarbon chains was observed at 42 °C. At the corresponding temperature

Table 1. Characterization of Various DPPC-Liposomes Used in This Study

	SUV	LUV	REV
Diameter/nm	40	345	1800
Entrapping efficiency[a]/%	1.9±0.4	8.7±2.7	17.7±2.9
Captured volume[b] (l/mole of lipid)	2.8±0.7	8.4±2.6	13.6±1.8

a) mean±S.D. (n=6). b) mean±S.D. (n=3).

Table 2. Barrier Efficiency of DPPC-REV in Buffer at 4 °C

	Remaining/% (mean±S.D., n=3)		
	1 day	1 week	4 weeks
Calcein	99.2±0.2	93.9±2.7	90.7±3.5
Dextran	98.3±7.5	97.0±5.5	98.2±8.3

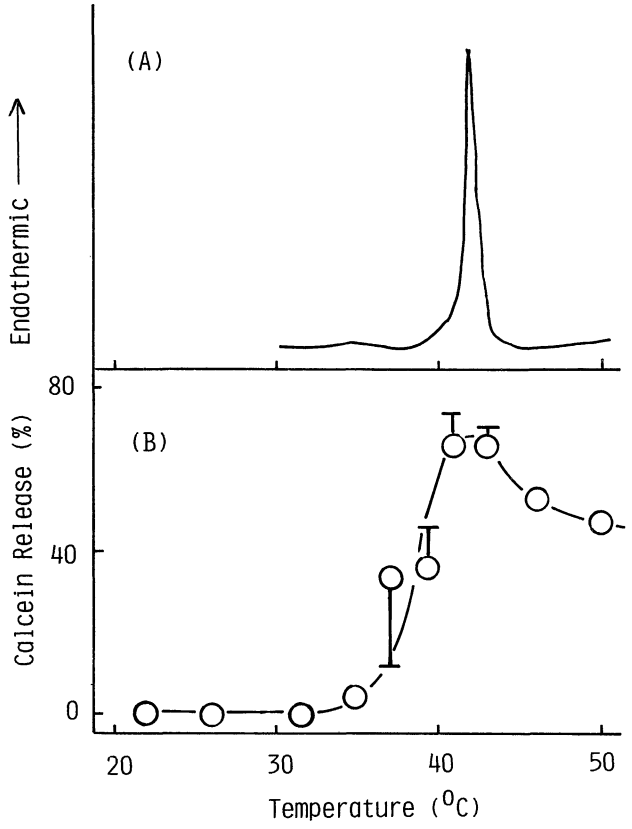

Fig. 1. Relationship between phase-transition temperature of DPPC-REV and calcein release (%) for 2 min incubation. (A): differential scanning calorimetry thermogram. (B): calcein release (%). Each point represents mean±S.D. (n=4—5).

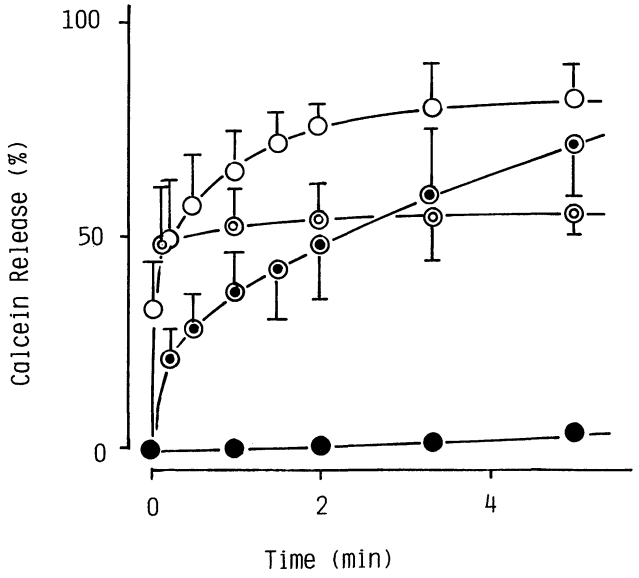

Fig. 2. Time course of calcein release from DPPC-REV. ●, 34.5°C; ◉, 39.3°C; ○, 41.0°C; ◎, 46.0°C. Each point represents the mean±S.D. (n=4—5).

the liposome showed maximum release. The time course of calcein release from DPPC-REV is shown in Fig. 2. The initial rate of calcein release seemed to become faster as the incubation temperature become higher; the rate at 46 °C was the highest measured, while the total amount of calcein released during 2 min of incubation became largest at 41 °C, near the phase-transition temperature, instead of 46 °C. At 46 °C, calcein release plateaued at a levels (ca. 50% of entrapped calcein) after 1 min of incubation. The drastic release observed during the initial stage at 46 °C would be due to the temporary experience of the optimum temperature of calcein release, (that is, the phase-transition temperature of the membrane phospholipid), during a temperature rise from room temperature to 46 °C. The saturation phenomenon regarding release % after 1 min of incubation at 46 °C suggests that the liposome again came to have a high barrier efficiency at a higher temperature than the transition temperature.

The Temperature-Sensitivity of Calcein Release Depending on the Preparation Method of the Liposomes. The calcein release % from liposomes prepared by several methods is shown in Fig. 3. EggPC-REV, whose phase-transition temperature is below 0 °C, did not practically make calcein release within experimental temperature range (24—47 °C), while DPPC liposomes prepared by any method showed (more or less) a temperature-sensitivity in calcein release near 40 °C. The release % of

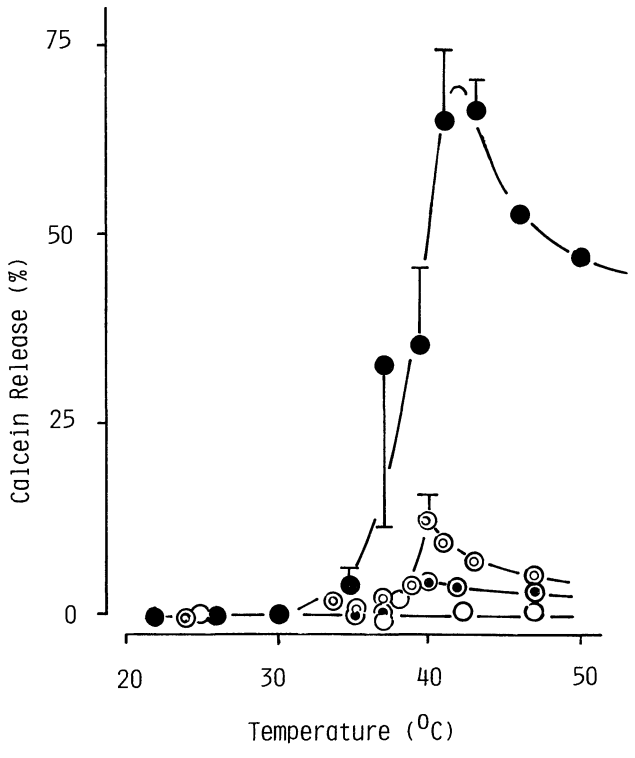

Fig. 3. Temperature dependence of calcein release from various types of liposomes. ○, eggPC-REV; ●, DPPC-REV; ◎, DPPC-LUV; ◉, DPPC-SUV. Each point represents mean±S.D. (n=4—5).

calcein was dependent on the method of liposome preparation; calcein release % from REV was the highest of all, followed by LUV, and then SUV.

Dextran Release from REV. Concerning DPPC-REV, which had showed the highest temperature-sensitivity for the release of calcein adopted as a model compound for low-molecular-weight drugs, the behavior of dextran release as a model compound for high-molecular-weight drugs was studied. The results are given in Figs. 4 and 5. As shown in Fig. 4, REV showed the maximum release (%) of dextran near 40 °C, similar to the case of a low-molecular-weight compound: that is, REV was found to possess the characteristics of temperature-sensitivity, even for the release of a high-molecular-weight compound. As shown in Fig. 5, the time course of dextran release from REV was also similar to that of calcein; that is, an initial high release followed by a dull release was observed. On the whole, the release % of dextran was smaller than that of calcein. For a high-molecular-weight compound, a saturation phenomenon was observed during its release, even at optimum temperature. The maximum amount of dextran released at the transition temperature was only 20% of encapsulated amount, while that of calcein was 70—90%. These observations suggest the inhomogeneity of the liposome in size and/or property; that is, the coexistance of both vesicles (permeable and inpermeable) for macro molecules, even at the transition temperature.

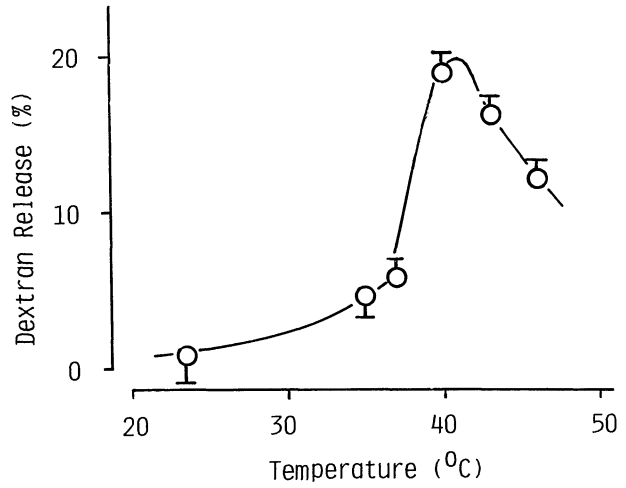

Fig. 4. Temperature dependence of dextran release from DPPC-REV. Each point represents mean±S.D. (n=4—5).

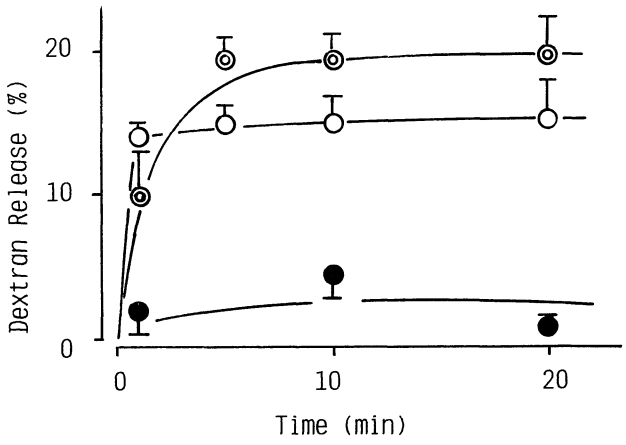

Fig. 5. Time course of dextran release from DPPC-REV at several temperatures. ●, 37 °C; ◎, 40 °C; ○, 42 °C. Each point represents the mean±S.D. (n=4).

Caracteristics of the Temperature-Sensitive Membrane Permeability. A pattern analysis of the time course of calcein release from DPPC-REV was carried out. In Fig. 6, the time course of the remaining percent of calcein in REV at 34.5 °C is shown on a logarithmic scale. At 34.5 °C the rate of calcein release was extremely slow and obeyed the first-order efflux. At 39.7 or 41.0 °C (near phase transition temperature) the efflux pattern seemed to have two or more phases; that is, an initial high efflux followed by a slow efflux was observed. These observations suggest that these liposomes must be inhomogenous in the size and/or property. In order to confirm the above suggestion, these liposomes were tentatively fractionated into 3 groups by centrifugation as S-REV with 100 nm of average diameter, M-REV with 2000 nm and L-REV with 3000 nm; the calcein efflux from each fractionated liposome was tested (data not shown). The calcein release rate was of the order of L-REV, M-REV, and S-REV. The polyphase of the efflux pattern for calcein release from the liposomes would be mainly due to the polydispersity of

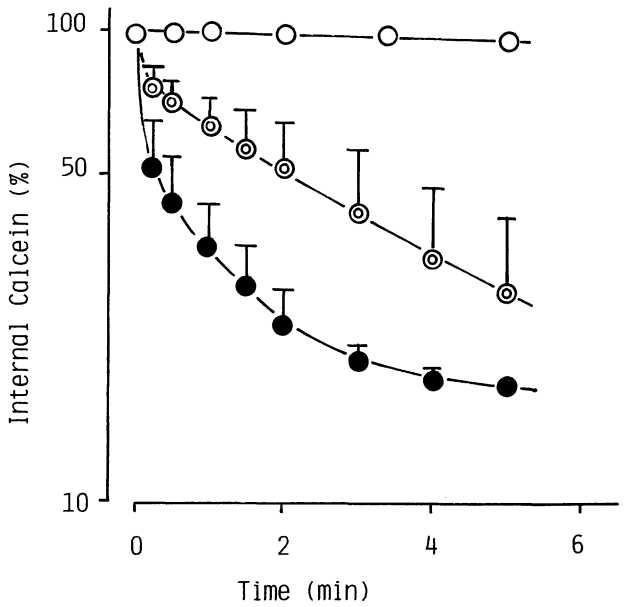

Fig. 6. Time course of remaining calcein (%) in DPPC-REV in logarithmic scale at several incubation temperatures. ○, 34.5 °C; ◎, 39.3 °C; ●, 41.0 °C. Each point represents the mean±S.D. (n=4).

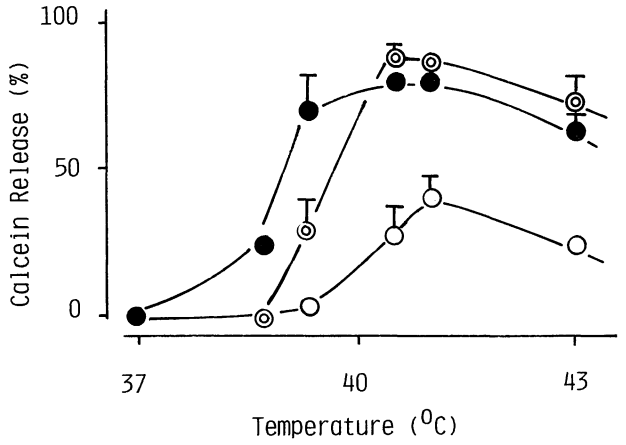

Fig. 7. Effect of liposome size on temperature-dependent calcein release. ●, L-REV; ◎, M-REV; ○, S-REV. Each point represents the mean±S.D. (n=3-4).

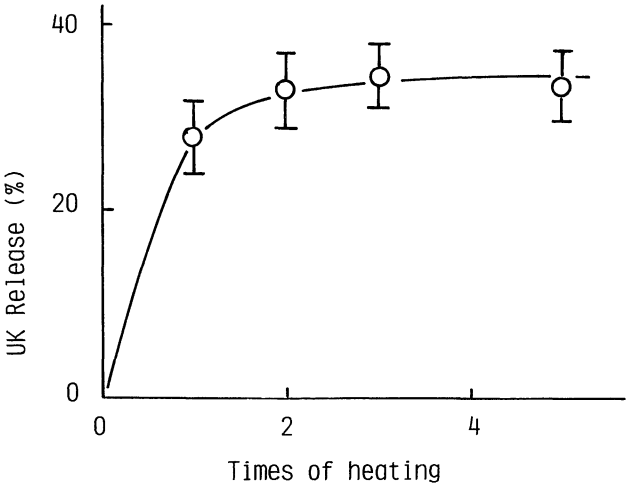

Fig. 8. Influence of the repeated heating on the release of urokinase from DPPC-REV. Each point represents mean±S.D. (n=3).

the liposomes in relation to size. In Fig. 7, the temperature dependence of calcein release for each fraction of the liposomes is shown. The temperature at which calcein release starts was found to shift to a lower temperature as the liposome size became larger. The maximum release % of L-REV and M-REV was almost the same as each other. The release % of S-REV was about half that of M-REV and L-REV. This observation also shows that the liposomes, which are registant for calcein release even at their transition temperature, somewhat exist in the liposome preparations, especially in S-REV.

Urokinase Release by Repeated Heating. One-minute heating of REV contaning urokinase was repeated several times at 5 min intervals. In Fig. 8, the urokinase release % is plotted against the number of repeating times. About 28% of the urokinase release was observed after the first 1 min of heating. Urokinase release upon a second heating was only 5%. After a third heating, no further urokinase release was observed. These results show that drug release from the temperature-sensitive liposome came to completion within two or so minutes heating, as is predicted by Fig. 2 for low-molecular-weight compound, or Fig. 5 for high-molecular-weight compound. Furthermore, these results suggest that there exist about 60% liposomes which are inpermeable for urokinase, even at the transition temperature.

Effect of the Molecular Weight of Encapsulated Compounds on Liposomal Permeation. The releases of several compounds with different molecular weights after 1 min incubation at 43 °C were plotted against the molecular weight in logarithmic scale, as shown in Fig. 9. The plotts fell into one curve according to their molecular weights, independent of the types of compounds: that is, low-molecular-weight organic compounds, proteins, polysaccarides. These results show that the releases of entrapped compounds from temperature-sensitive liposomes near their transition temperatures could be primarily governed by their molecular weight, in spite of the variety of drugs.

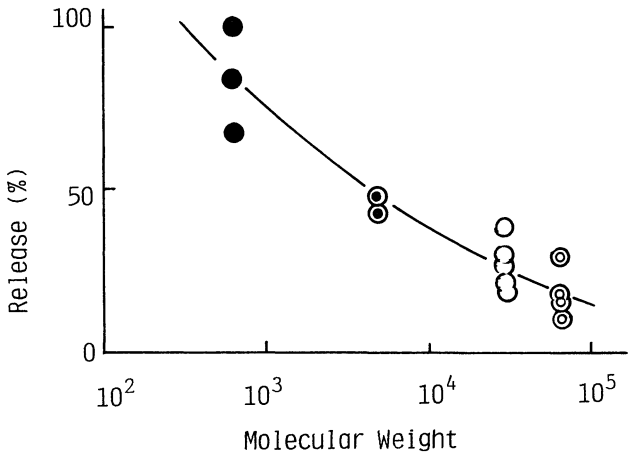

Fig. 9. Molecular weight dependence on the release of model drugs from DPPC-REV. ●, calcein; ◉, inulin; ○, urokinase; ◎, dextran.

Discussion

The initial release rate of both a low-molecular-weight compound (calcein) and a high-molecular-weight compound (dextran) from liposomes became larger as the incubation temperature was elevated within the experimental temperature limit (34.5—46 °C). At temperatures higher than the phase-transition temperature, the release amount attained saturation within a few minutes of incubation. Regarding calcein release at 46 °C, saturation was achieved within 1 min of incubation, and the released calcein upon saturation was about 50% of the calcein entrapped in the

liposome. No further release was added, even after long-time incubation. These observations suggest that the high initial rate at a temperature above the phase-transition temperature might be brought about when the liposome experiences the phase-transition temperature while increasing the temperature from low temperature to a fixed temperature; the liposome might again have a high barrier efficiency after the temperature exeeds the phase transition temperature.

Even at the transition temperature, a saturation phenomenon was also observed in the case of the release of a macromolecule. Saturation was achieved within a few minutes. The amount of release at saturation was dependent on the molecular weight of the compounds entrapped, and was 20—40% of the total macromolecules entrapped. No release exeeding 20—40% was observed, even after long-time incubation. These phenomena might be due to the polydispersity of the liposome size and properties: that is, 2 or more species of liposomes (one liposome could be perturbed enough large to bring about membrane release of macromolecules and the others not,) coexisted. Strictly speaking, even for a low-molecular-weight compound, no 100% release occured at all; thus, about 10% of the liposome was impermeable, even at the transition temperature. Judged from the results that the extent of temperature-sensitivity of drug release from a vesicle was dependent on the method of liposome preparation as well as on fractionation by centrifugation of the liposomes, larger liposomes seemed to generate a higher-temperature-sensitive drug release, suggesting that the extent of structural deformation of the membrane at the phase-transition temperature would vary among liposomes, especially depending on the liposome size.

Regarding the structural deformation of the membrane at the phase-transition temperature, which brings about an abrupt lowering of the membrane barrier efficiency, several hypotheses have been reported: (1) the apearance of a boundary between the gel phase and the liquid crystalline phase accompanying lateral phase separation,[16,17] (2) a change in the lateral compresibility between adjacent phospholipid molecules with an increase in membrane fluidity,[18] and (3) the formation of statistical pores by the rotation of phospholipid molecules with isomerization.[19] Since these hypotheses are based on the behavior of low-molecular-weight compound or ions, it is difficult to restrict the mechanism for such a rapid permeation of a high-molecular-weight compound as observed in our present experiments. In any case, it should at least be concluded that a large perturbation, which enables even a rapid permeation of macromolecules, must arise in a membrane on the occasion of a phase transition; also, the degree of perturbation must vary with the properties of the liposomes, especially regarding their size.

This work was supported by a Grant-in Aid for Scientific Research No. 60460218 (M.U.) and No. 6187009 (I.H.) from the Ministry of Education, Science and Culture.

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