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Improvement of Encapsulation Efficiency of Water-Soluble Drugs in Liposomes Formed by the Freeze-Thawing Method

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In order to improve the encapsulation efficiency (*EN*%) of a water-soluble drug in liposomes formed by the freeze-thawing (FT) method, the influence of preparation conditions was examined in detail using L-asparaginase (A-ase) as a model drug. The results were compared with those obtained by the thin film (hydration) method. It became clear that lower ionic strength in the suspension medium, gentle shaking after thawing, higher concentration of phospholipid and a relatively low concentration of A-ase were preferable to obtain higher *EN*% in the FT method.

To confirm these results, other drugs of various molecular weights, cyanocobalamin, inulin and glucose, were encapsulated under controlled preparation conditions so as to obtain high *EN*%. High *EN*% values of from 40% to 60% (evaluated by the gel filtration method) were obtained.

The influence of the phospholipid composition was also examined using phosphatidylcholine (PC), a mixture of PC and stearylamine, and a mixture of PC and dicetylphosphate. All of them gave lower *EN*% than yolk phospholipid (YPL). The mechanism of liposome generation is discussed on the basis of the results obtained.

Keywords——liposome; freeze-thaw; preparation condition; encapsulation efficiency; L-asparaginase; cyanocobalamin; inulin; glucose; gel filtration

Since the discovery of multilamellar structure in liposomes by Bangham *et al.*,[1] such vesicles have been studied by many researchers. In the pharmaceutical field, the potential utility of liposomes as a drug carrier was recognized and many investigations have been performed from this point of view.[2] However, most of the preparation methods presented to date have used some organic solvents or detergents and the procedures are rather complicated. These limitations have restricted the practical application of liposomes. Thus, we developed two novel methods, the freeze-thawing (FT) method[3] and the freeze-drying (FD) method,[4] which need not use any organic solvent or detergent, and seem to be practical for technical production.

In the preparation of liposomes, it is important to obtain high encapsulation efficiency (*EN*%),[5] and this was investigated by several researchers in the case of the thin film method[6] and another method.[7] It is known that *EN*% depends upon the ionic strength of the aqueous medium,[6] the preparation method of the phospholipid suspension, the time of hydration and so on,[5] but little systematic research has yet been performed.

Thus, to develop the FT method further, L-asparaginase was used as a model drug and optimal preparation conditions were investigated. The effects of ionic strength, shaking conditions after thawing, phospholipid concentration, A-ase concentration and phospholipid composition on the properties of the liposomes were investigated and compared with those in the case of the conventional thin film method.

The optimal conditions thus identified were applied to other drugs having various molecular weights, cyanocobalamin (molecular weight, 1350), inulin (about 5000) and glucose (180). The results obtained are discussed in relation to the mechanism of liposome generation by the FT method.

Experimental

Materials——Yolk phospholipid mixture (YPL) was extracted from egg yolk as described in the previous paper.[3] L-Asparaginase (A-ase) was purchased from Kyowa Hakko Kogyo Co., Ltd. (Tokyo, Japan). Cyanocobalamin (CN-cbl) was purchased from Roussel Uclaf Japan (Tokyo, Japan). [U-^{14}C] Glucose, [methoxy-^{14}C] inulin and L-α-dipalmitoyl [2-palmitoyl-9,10-^{3}H] phosphatidylcholine ([^{3}H] DPPC) were purchased from Japan Radio Isotope Association (Tokyo, Japan); their specific activities were 4.4 mCi/mmol, 41.9 mCi/g and 60.0 Ci/mmol, respectively. The manufacturer's estimates of radiochemical purity, 99% for [^{14}C] glucose, 99% for [^{14}C] inulin and 98% for [^{3}H] DPPC, were confirmed by gas liquid chromatography and thin layer chromatography. Other chemicals used were of the highest grade commercially available.

Preparation of Liposomes——Typical preparation methods are described below.

1) FT Method: a) A-ase: YPL suspensions whose YPL concentrations were from 5% to 25% were obtained by dispersing YPL in 0.05 M Tris–HCl buffer (pH 8.0) using an ultrasonic disintegrator (model 300, Ultrasonic Ltd., London, U.K.) in an ice bath under nitrogen gas flow. This buffer solution was used (denoted as Tris buffer hereafter) since A-ase is known to be stable at around pH 8. This suspension was mixed with an A-ase solution and filtered through a membrane filter (pore size 0.8 μm). Each 2 ml fraction of the filtrate was taken in a 5 ml glass ampule, frozen at −20 °C and kept at this temperature for 24 h. The frozen mixture was thawed at room temperature, and subsequently shaken with a Vortex mixer (1000 rpm) for 20 min.

b) CN-cbl, Glucose and Inulin: The above Tris buffer solution was replaced with distilled water to prepare liposomes of these drugs.

Liposomes obtained by the FT method are denoted as FT liposomes, hereafter.

2) Thin Film Method: Liposomes were prepared by the conventional thin film method originated by Bangham *et al.*[1] with a slight modification as follows; YPL (from 0.25 to 2 g) was dissolved in 20 ml of chloroform in a round-bottomed 300 ml flask, then the chloroform was evaporated using a rotary evaporator. A thin film of YPL was formed on the surface of the flask. The residual trace solvent was removed under nitrogen gas flow. The thin film of YPL was hydrated with an appropriate volume of a drug solution (A-ase in Tris buffer, other drugs in distilled water) and subsequently shaken with Vortex mixer. The liposomes prepared by this method are denoted as HY liposomes.

When the effect of shaking conditions was investigated, both types of liposomes were agitated with a Vortex mixer, a homomixer (Ultra-Turrax, TP 18-10) or a probe type sonicator.

Analytical Method——1) A-ase: A-ase activity was determined by the method described in the previous paper[3] using L-asparagine as a substrate.

2) CN-cbl: A 1 ml aliquot of liposomal suspension was solubilized by the addition of 1 ml of 10% Triton X-100 solution. This solution was diluted with distilled water, and the absorbance at the wavelength of 550 nm was measured.

3) [^{14}C] Glucose, [^{14}C] Inulin and [^{3}H] DPPC: The sample solution (1 ml) was mixed well with 10 ml of scintillation medium (Univer-Gel II, PPO, Bis MBS, nonionic surfactant in xylene, Nakarai Chemicals Co., Kyoto, Japan), and the ^{3}H and ^{14}C contents were measured in a liquid scintillation system (Packard, Tri-Carb 460C).

Calculation of Encapsulation Efficiency——1) A-ase: $EN\%$ was calculated by means of Eq. 1 from the difference of activities between solubilized liposomes treated with Triton X-100 (total activity, denoted as TA) and those without Triton X-100 treatment (free activity, FA) as described in the previous paper.[3]

$$EN_a\% = 100 \times (TA - FA)/TA \quad (1)$$

2) CN-cbl, Glucose and Inulin: For these three drugs, gel filtration was performed as described previously using a Sepharose 4B column.[3] Encapsulation percentage ($EN_g\%$) was calculated according to Eq. 2.

$$EN_g\% = 100 \times A_e/(A_e + A_f) \quad (2)$$

Here, A_e and A_f mean the amount of drug in the encapsulated fraction and in the free drug fraction, respectively.

Evaluation of Particle Size Distribution——1) Electron Microscopy: Electron micrographs of liposomes were taken and the diameter of individual particles was measured as described previously.[3]

2) Turbidity ($A_{1\%}$): A liposome preparation was diluted with Tris buffer so that the concentration of YPL was 0.025%, and the absorbance (A) of the diluted liposomal suspension was measured at 600 nm at 25 °C in a 1 cm cuvette by using a spectrophotometer. $A_{1\%}$, which corresponds to the absorbance of a 1% YPL suspension, was calculated by multiplying A by 40 (=1/0.025).

Results and Discussion

Influence of Electrolytes in the Suspension Medium

Previously, we compared two novel methods for preparing liposomes, the freeze-thawing (FT) method[3] and the freeze-drying (FD) method,[4] with the conventional thin film method

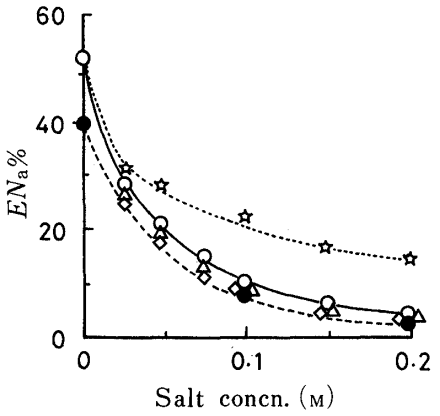

Fig. 1. Influence of Salt Concentration on the Encapsulation of A-ase in Liposomes

Lipid phase, YPL (50 mg/ml); suspension medium, Tris buffer containing A-ase (10 IU/ml) and salt (the concentration is shown on the abscissa).

Open symbols, FT method; closed symbols, thin film method.

○●, NaCl; ☆, KCl; △, $CaCl_2$; ◇, $MgCl_2$.

proposed by Bangham[1] using A-ase as a model drug. The encapsulation percentage ($EN\%$) of A-ase was very low when it was prepared according to Bangham. However, altering the suspension medium from buffered saline (0.05 M Tris–HCl buffer, 0.15 M NaCl)[4] to Tris buffer (0.05 M Tris–HCl buffer) resulted in a higher $EN\%$.[3] As these phenomena seemed to be caused by the difference of ionic strength, the effect of electrolyte concentration in the suspension medium on $EN\%$ was examined using univalent electrolytes (sodium chloride and potassium chloride) and divalent electolytes (magnesium chloride and calcium chloride). As shown in Fig. 1, the $EN\%$ rapidly decreased as the concentration of electrolytes increased in the aqueous phase irrespective of the kind of electrolyte. In the case of sodium chloride, the effect was also investigated in the thin film method. It was shown that an increase in sodium chloride concentration also resulted in a rapid decrease of $EN\%$ in the thin film method.

Reeves and Dowben showed that large unilamellar or oligolamellar vesicles were obtained when distilled water was used as an aqueous phase in the thin film method.[6a] Tyrrell *et al.* showed that the internal aqueous phase volume in liposomes increased with decrease of the ionic strength.[6b] Therefore, the results in Fig. 1 indicate that the changes of $EN\%$ were qualitatively in agreement with their description in both the FT and the thin film method.

Thus, to increase $EN\%$, sodium chloride was removed from the suspension medium of the conventional thin film method by Bangham; the modified method is denoted as the HY method hereafter.

Influence of Shaking Condition

The conditions of shaking after thawing seemed to have a considerable influence on the properties of the liposomes.[8] Figure 2 shows the $EN\%$ of A-ase and $A_{1\%}$ of liposomes prepared under various shaking conditions for both FT and HY preparations.

In Fig. 3, particle size distributions determined by electron microscopy are shown for liposomes whose $A_{1\%}$ values were 12.0% and 1.8%, as examples. Liposomes of larger $A_{1\%}$ value have a larger mean particle size and *vice versa.*

Recently, we showed by use of the sedimentation method that the mean diameters (D) of liposomes increased linearly with increase of $A_{1\%}$.[9] For more than ten liposome formulations prepared by various methods, the following relationship held in the $A_{1\%}$ range from 0 to 40; the correlation coefficient was more than 0.9.

$$D=0.031\times A_{1\%}+0.34 \qquad (r=0.91)$$

The average diameter obtained from Fig. 3 was about half of that calculated from the above equation, but the difference may arise from the method adopted to evaluate the average diameter. Namely, the former was measured in the dried state whereas the latter was measured in the dispersed state in water. Thus, $A_{1\%}$ can be regarded as an index of liposomal

particle size.

Both $EN\%$ and $A_{1\%}$ of the liposomes prepared by ordinary shaking with a Vortex mixer were nearly equal to those prepared by shaking with the homomixer, but the use of glass beads (radius 1 mm, 1 g in 2 ml of liposome suspension) with the Vortex mixer reduced them considerably. The effect of a probe type sonicator was the largest. $EN\%$ and $A_{1\%}$ decreased rapidly as the sonication time became longer. Huang described that the trapped volume of liposomes was decreased by ultrasonic disintegration.[10] Saunders *et al.* showed that the first few minutes of shaking by ultrasonic disintegration had the largest effect on average vesicle size.[11] These findings are coincident with the results shown in Fig. 2b.

Figure 2a shows that the tendencies observed for the HY method were also found for the FT method. However, a comparison of $A_{1\%}$ and $EN\%$ values obtained by the two methods indicated that FT liposomes generally have a larger $EN\%$ but smaller $A_{1\%}$, if the shaking conditions are the same. The relationships between $A_{1\%}$ and $EN\%$ are shown for FT and HY liposomes in Fig. 4. $EN\%$ increased as $A_{1\%}$ increased in both cases. This means that as the particle size increases, more drug is encapsulated. However, $EN\%$ of FT liposomes is always larger than that of HY liposomes if the $A_{1\%}$ values of the two liposomes are equal to each other. This suggests that the FT method is a more effective method for a proteinous drug such as A-ase, because it gives a higher $EN\%$ if the particle size is the same. In the previous paper, we showed that the average $EN\%$ is larger in FT liposomes than in HY liposomes. The results in Fig. 4 confirm that this is also true for liposomes having various particle sizes. We presumed that the difference in $EN\%$ is a result of the difference of generation mechanism of the liposomes in the two methods, as discussed previously.[3] That is, in the case of HY preparation, liposomes probably occur successively on the surface between the phospholipid

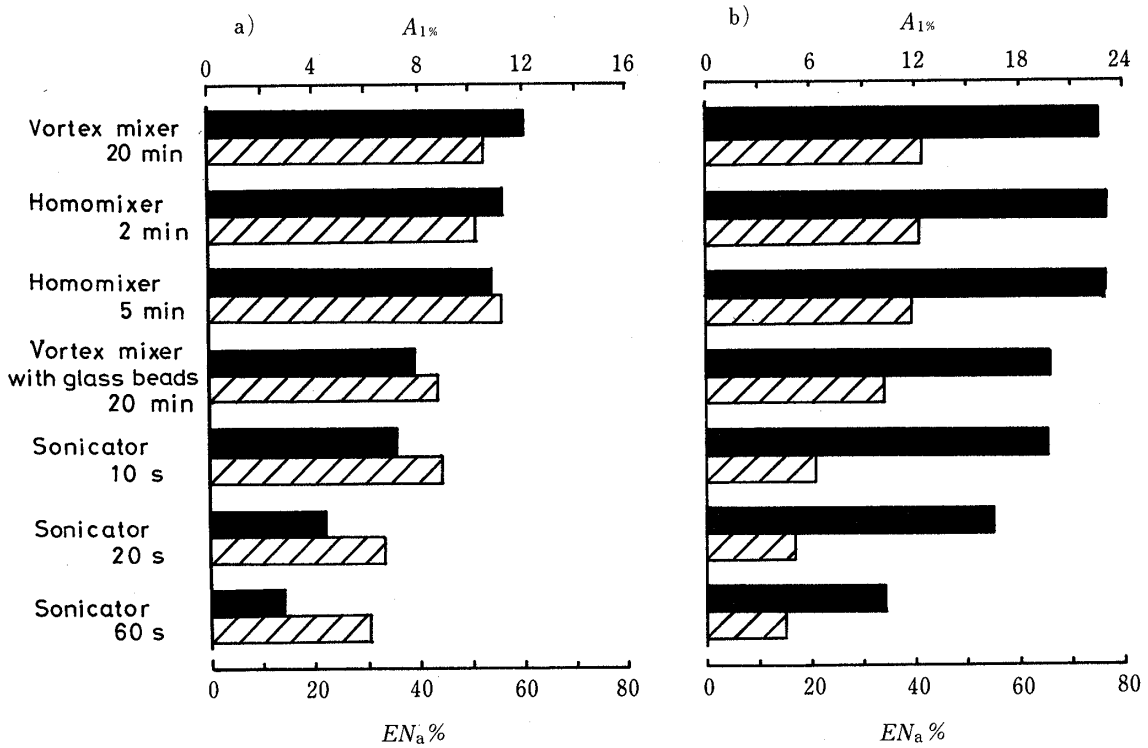

Fig. 2. Influence of Shaking Conditions on the Encapsulation of A-ase and Turbidity

Lipid phase, YPL (50 mg/ml); suspension medium, Tris buffer containing A-ase (10 IU/ml).
a) FT liposomes; b) HY liposomes.
▨, $EN_a\%$; ■, $A_{1\%}$.

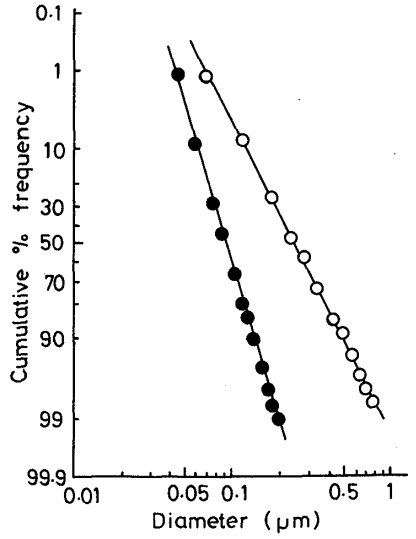

Fig. 3. Particle Size Distribution Determined by the Electron Micrographic Method for Liposomes Having Various $A_{1\%}$ Values

●, $A_{1\%}=1.8$; ○, $A_{1\%}=12.0$.

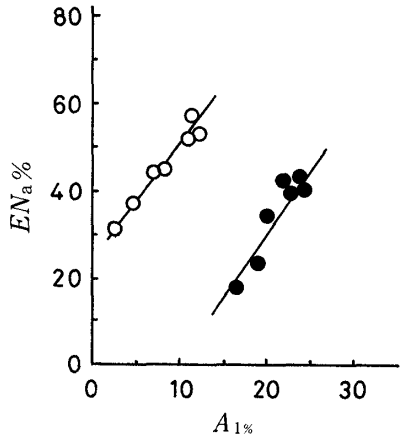

Fig. 4. Relationship between $A_{1\%}$ and EN_a% for FT Liposomes and HY Liposomes

○, FT liposomes; ●, HY liposomes.

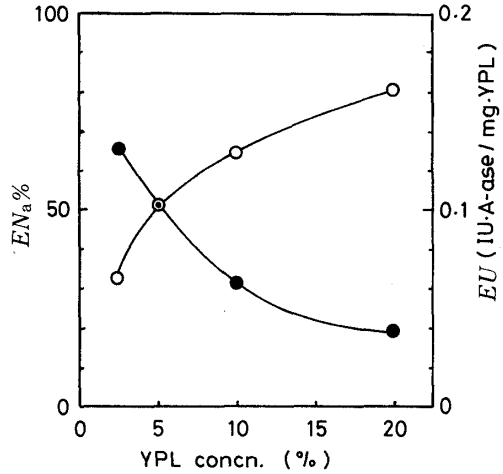

Fig. 5. Influence of YPL Concentration on the Encapsulation of A-ase in FT Liposomes

Lipid phase, YPL (the concentration is shown on the abscissa); suspension medium, Tris buffer containing A-ase (10 IU/ml).

○, EN_a%; ●, EU (the amount of encapsulated A-ase per 1 g of YPL).

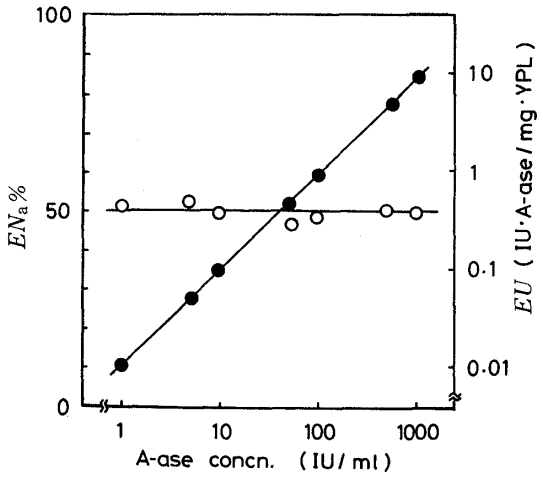

Fig. 6. Influence of A-ase Concentration on the Amount of A-ase Encapsulated in FT Liposomes

Lipid phase, YPL (50 mg/ml); suspension medium, Tris buffer containing A-ase (the concentration is denoted in the abscissa).

○, EN_a%; ●, EU (the amount of encapsulated A-ase per 1 g of YPL).

film and continuous aqueous phase containing A-ase. When the generation of liposome proceeds, more A-ase in the continuous phase is encapsulated in it, consequently the concentration of A-ase in the continuous phase is presumed to decrease successively. As the concentration of A-ase in the continuous phase greatly affects the EU values, as shown in Fig. 6, those of individual liposomes occurring later are presumably lower. On the other hand, in the case of FT preparation, liposomes are prepared simultaneously in all places in the phospholipid suspension in the frozen state, and this gives a high EN%, as represented previously.[3] The concentration of A-ase is high and uniform, so almost all FT liposomes seem to be prepared in an environment of higher A-ase concentration than HY liposomes on the

whole, consequently resulting in higher *EN*%.

Influence of YPL Concentration

The influence of YPL concentration on *EN*% is shown in Fig. 5. *EN*% increased from 30% to 80% as the YPL concentration was increased from 2.5% to 20%. However, the amount of encapsulated A-ase per one gram of YPL (denoted as *EU*) decreased as the YPL concentration increased. This means that the fraction of YPL taking part in encapsulation decreases as YPL increases.

Influence of A-ase Concentration

The dependency of *EU* on the A-ase concentration is shown in Fig. 6. *EU* increased linearly with A-ase concentration. However, *EN*% did not change and was about 50% irrespective of the A-ase concentration. As shown previously, A-ase once encapsulated in liposomes scarcely leaked from the inner layer even under the influence of osmotic pressure.[3] Thus, the following is inferred to occur at the stage of the preparation process.

As A-ase is very soluble in the aqueous phase and insoluble in the lipid phase, it probably exists in the aqueous phase before the generation of liposomes by thawing. Therefore, *EN*% depends upon the amount of the aqueous phase enveloped in the phospholipid agglomerate during the thawing process. The result that *EN*% scarcely changed with the concentration of A-ase suggests that the enveloped aqueous phase is almost the same irrespective of A-ase concentration. A-ase itself probably has little effect on the physical properties of vesicles such as particle size, distribution, shape, *etc.* If A-ase had much effect on these properties, then *EN*% would be expected to change, because *EN*% was considerably influenced by the particle size of vesicles, as shown in Fig. 4.

Application to Other Water-Soluble Drugs

From the example of A-ase, it was clarified that the following items are necessary to obtain liposomes of high *EN*% by the FT method.

(1) The ionic strength of the suspension medium (aqueous phase) should be as low as possible.
(2) Intense shaking such as ultrasonication should be avoided. Gentle shaking in a Vortex mixer is preferable.
(3) YPL concentration should be increased as much as possible and the ratio of drugs to YPL should be decreased.

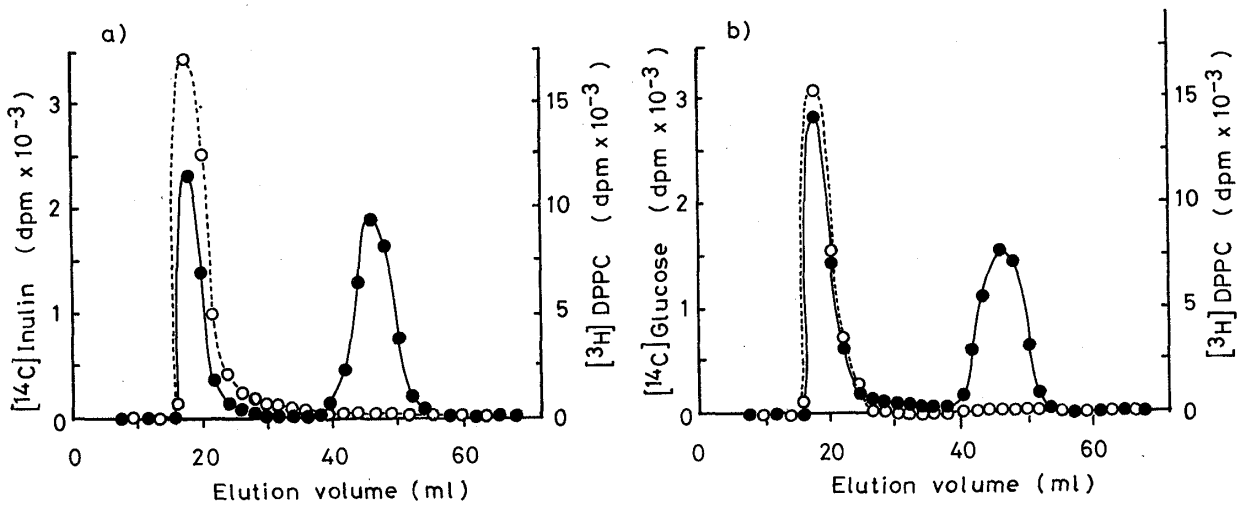

Fig. 7. Gel Filtration Profile of FT Liposomes Containing [^{14}C] Inulin (a) and [^{14}C] Glucose (b)

○, [^{14}C] inulin (a) and [^{14}C] glucose (b) ●, [^{3}H] DPPC.

TABLE I. Comparison of Encapsulation Efficiency[a] of Various Water-Soluble Drugs

Method	FT	FT	HY
Suspension medium	Dist. water	Buffered saline[b]	Dist. water
Glucose	53.6	2.3	53.9
CN-cbl	62.1	6.5	59.2
Inulin	44.6	5.1	42.0

Each 1 ml of liposome suspension contains 50 mg of YPL and 1 mg of a drug. *a*) $EN_g\%$ estimated from Eq. 2. *b*) 0.05 M Tris–HCl buffer (pH 7.4) containing 0.15 M NaCl. Each value represents the mean of at least three experiments.

To confirm these conclusions, other water-soluble drugs having various molecular weights, CN-cbl, inulin and glucose, were encapsulated. In these cases, distilled water was used as the suspension medium to increase $EN\%$ (see conclusion (1)). The gel filtration method was adopted to determine $EN\%$ of these materials. Figure 7 shows the concentrations of [^{14}C] inulin and [^{14}C] glucose in each fraction collected by the gel filtration method. In this experiment, ^{3}H labelled DPPC was also used to examine how the vesicle materials interact with the drug materials. As shown, both [^{14}C] inulin and [^{14}C] glucose gave two peaks at elution volumes of around 20 ml and around 50 ml. The former and the latter peaks corresponded to encapsulated drug and free drug, respectively. Thus, from the amounts of the drug in each peak, $EN_g\%$ was calculated using Eq. 2.

The peak of ^{3}H was coincident with that of ^{14}C of encapsulated drugs and did not appear elsewhere (Fig. 7a). This means that almost all of the phospholipid exists as agglomerates (namely, it is used for liposome preparation) and scarcely any exists as molecular phospholipid. These suggestions were supported by the similar tendency observed in [^{14}C] glucose liposome (Fig. 7b).

The $EN\%$ values of FT liposomes determined by the gel filtration method are summarized in Table I in comparison with those of HY liposomes. The $EN\%$ of the FT liposomes prepared at higher ionic strength is also shown for comparison. It is clear that electrolyte greatly reduced $EN\%$ in all cases. Therefore, the conclusions based on the A-ase investigation seem to have generality. The three model drugs were all effectively encapsulated to extents of more than 45% by the FT method without any electrolytes. These high values of $EN\%$ for all drugs confirm the utility of the FT method.

Effect of the Components in the Lipid Phase

The phospholipid components were expected to affect the properties of the liposomes. Phosphatidylcholine (PC) is regarded as a main component of YPL, so the encapsulation of CN-cbl was performed using PC as the lipid phase. In this experiment, gel filtration and ultrafiltration[12] were adopted to determine $EN\%$ values, because PC/CN-cbl liposomes did not pass through the gel filtration column owing to the large particle size. Ultrafiltration was carried out through a membrane (UK-50, Toyo Roshi, Japan) at a pressure of 2 kg/cm^2 of nitrogen gas. The effluent was collected in 2 ml portions, the concentration of CN-cbl was determined, and the encapsulation percentage ($EN_u\%$) was calculated by means of Eq. 3.

$$EN_u\% = 100 \times (C_{sol} - C_{lip})/C_{sol} \quad (3)$$

Here, C_{sol} and C_{lip} mean the equilibrium CN-cbl concentrations of the solution (without YPL) and the liposomal suspension, respectively.

Figure 8 shows an example of the changes in CN-cbl concentration. The rather low initial value was presumably due to the water remaining in the apparatus beforehand, but the value subsequently reached equilibrium level. $EN_u\%$ was calculated from the equilibrium values using Eq. 3.

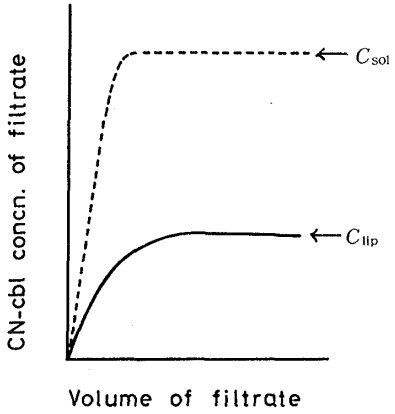

Fig. 8. Schematic Illustration of CN-cbl Concentration in Each Portion of the Ultrafiltrate

Solid line, liposomal suspension; broken line, CN-cbl solution (without YPL).

Table II. Influence of Lipid Compositions on the Encapsulation Efficiency of CN-cbl in FT Liposomes

Lipid composition	Encapsulation efficiency EN_g%	EN_u%
PC	—	48.5
PC+SA (10:1)[a]	43.8	50.6
PC+DCP (10:1)[a]	34.7	42.5
YPL	62.1	74.7

Each 1 ml of liposome suspension contains 50 mg of phospholipid and 1 mg of CN-cbl. *a*) Molar ratio. Each value represents the mean of at least three experiments.

PC gave rather lower EN% than YPL, as shown in Table II. YPL contains many components other than PC, and so another lipid, stearylamine (SA) or dicetylphosphate (DCP), was mixed with PC and encapsulation was performed. EN% did not change with the addition of SA, but was reduced by the addition of DCP. It is well known that SA bears a positive charge and DCP bears a negative charge. The above results suggest that encapsulation efficiency is greatly affected by the nature of the components in the lipid phase.

A comparison of EN% values obtained by the gel filtration method (EN_g%) and the ultrafiltration method (EN_u%) showed that the latter was always about ten percent higher than the former. This tendency was also observed with glucose and inulin (these data are not shown) and a good linear relationship was obtained between EN_g% and EN_u%. The reason why higher values were obtained by ultrafiltration is not cleare, but this seems to be an intrinsic feature of the method, and is therefore of interest.

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