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Evaluation of a New Liposome Preparation Technique, the Freeze-Thawing Method, Using L-Asparaginase as a Model Drug

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A new and simple technique for the preparation of liposomes, the freeze-thawing method (FT method), was developed and evaluated for encapsulation efficiency by using L-asparaginase (A-ase) as a model drug. The encapsulation percentage of A-ase in liposomes ($EN\%$) was determined by three methods, *i.e.* the measurement of free and total activities of A-ase, the gel filtration method and the trypsin treatment method.

A-ase was encapsulated in FT liposomes with a higher efficiency than in liposomes prepared by the hydration method (HY liposomes), although the shape, the particle size distribution and the appearance of multilamellar structure of FT and HY liposomes were similar to each other. A-ase encapsulated in FT liposomes was resistant to proteolysis by trypsin treatment and to leakage by osmotic shrinkage.

The FT method has the following advantages over the HY method for the practical preparation of liposomes as drug carriers: the use of an organic solvent or a detergent is unnecessary, sterilization can be carried out with a membrane filter and the preparation technique is simple.

Keywords——liposome; preparation method; L-asparaginase; freeze-thaw; encapsulation efficiency; electron microscopy; gel filtration; trypsin treatment; particle size distribution

Since the discovery of lamellar structure in liposomes by Bangham *et al.*,[1] many researchers have studied liposomes extensively as a model of biological membranes or as a carrier material for drugs.[2,3] A part of the work has already reached the stage of practical pharmaceutical application.[4] However, in order for liposomes to be used more widely for therapeutic purposes, the preparation processes should satisfy the following standards: (1) a high degree of encapsulation of drugs can be attained; (2) organic solvent or detergent can be completely removed; (3) sterilization can be carried out easily; (4) the final product is obtainable by simple procedure; (5) preparation can be carried out on a large scale; (6) stability is good enough to guarantee the quality for an appropriate strorage period. So far, various methods such as the hydration thin film method,[1b] detergent removal method,[5] ether injection method,[6] and reverse-phase evaporation method[7] have been proposed by many researchers and their comparative properties and practical usefulness have been discussed.[8] However, almost all of these methods require the use of organic solvents or detergents and do not meet the above criteria.

In order to overcome the above problem, we have devised two novel methods which need not use any organic solvents or detergents, *i.e.* the freeze-drying (FD) method and freeze-thawing (FT) method.[9] Details of the FD method, and the results when applied to L-asparaginase were reported recently.[9b] As regards the FT method, the procedure was disclosed in detail in a Japanese patent in 1976.[9a] Thereafter Kasahara and Hinkle reported the application of the FT method to the encapsulation of a glucose transporting protein,[10] and more recently, Pick investigated this method in detail by using radioactive chemicals such as ions, small organic molecules and proteins.[11]

We investigated the utility of the FT method in this work using L-asparaginase as a model drug. The shapes and encapsulation efficiency of FT liposomes were examined by various methods, such as electron microscopy, gel filtration and trypsin treatment in comparison with those of liposomes prepared by the conventional method originated by Bangham *et al.*[1b]

Experimental

Materials——Phospholipid mixture (YPL) was extracted from egg yolk with 95% ethanol.[12] Ethanol was removed by evaporation under reduced pressure, and the residue was purified by washing with acetone.[5a] After removal of the acetone, obtained YPL was loaded into an air-tight bottle and stored under nitrogen at −20 °C in the solid state. The major components of YPL as determined by thin-layer chromatography (TLC) were phosphatidylcholine (75.5%) and phosphatidylethanolamine (16.5%). L-Asparaginase (Leunase injection, designated as A-ase) and trypsin were obtained from Kyowa Hakko Kogyo Co., Ltd. (Tokyo Japan) and from Sigma (U.S.A.), respectively. All other chemicals used were of the highest grade commercially available.

Preparation of Liposomes——FT Method: The procedure for liposome preparation by the FT method is shown in Fig. 1. First, 1 g of YPL was dispersed in 9 ml of 0.05 M Tris–HCl buffer, pH 8 (this buffer solution is abbreviated as Tris buffer hereafter), using a probe type sonicator (model 300, Ultrasonic Ltd., London, U.K.). This dispersion (YPL 10%) was mixed with an equal volume of A-ase solution (20 IU/ml) in Tris buffer, and the mixture was filtered through a membrane filter (pore size 0.45 μm). Then 2 ml of the filtrate was taken in a 5 ml glass ampule, frozen at −20 °C, and kept at that temperature for 24 h. The frozen mixture was thawed at room temperature and subsequently shaken with a Vortex mixer for 20 min. The liposomes obtained by this method were designated as FT liposomes.

HY Method: Liposomes were prepared by a conventional preparation method originated by Bangham *et al.*[1b] with slight modifications; 0.5 g of YPL was dissolved in 20 ml of chloroform in a 300 ml round-bottomed flask. The chloroform was evaporated off in a rotary evaporator under reduced pressure to leave a thin film of YPL on the surface of the flask. The residual trace solvent was removed under a nitrogen gas flow. The thin film of YPL was hydrated with 9.5 ml of A-ase solution (10.5 IU/ml) in Tris buffer and subsequently shaken in a Vortex mixer for 20 min. The liposomes prepared by this method were designated as HY liposomes.

Electron Microscopy——Liposomes were negatively stained with sodium phosphotungstate at pH 8, and observed with an electron microscope (Japan Electron Optics Lab. Co., Ltd., model JEM-100, Tokyo, Japan). The longer diameter of individual vesicles and that of the central water compartments were measured by using a particle size analyzer (Karl Zeiss, Germany).

Determination of L-Asparaginase Activity——Activity of Free A-ase: The activity of A-ase was determined by the method of Citri and Zyk[13] with a slight modification, using L-asparagine as a substrate. First, 0.25 ml of liposomal suspension, 1 ml of 0.16 M L-asparagine solution and 2.5 ml of Tris buffer were mixed, and incubated for 16 min at 37 °C. The reaction was terminated by the addition of 1 ml of 15% trichloroacetic acid. Then, 20 ml of 1 N sodium hydroxide solution was added and liberated ammonia was determined by using an ammonia electrode (Horiba, No 5002-05T, Kyoto, Japan). The A-ase activity liberating 1 μmol of ammonia per min at 37 °C is defined as one unit (1 IU). The activity of A-ase determined as described above is referred to as free activity (*FA*) hereafter.

Total Activity (*TA*) of A-ase: The A-ase encapsulated in liposomes does not react with L-asparagine present in the outer solution. In order to evaluate the *TA* of A-ase existing both in liposomes and in the outer solution, liposomes were lysed with Triton X-100. Thus, one-fifth of the Tris buffer (0.5 ml) in the above *FA* measurement was replaced by 0.5 ml of 10% Triton X-100 solution, and other procedures were performed similarly. The activity of A-

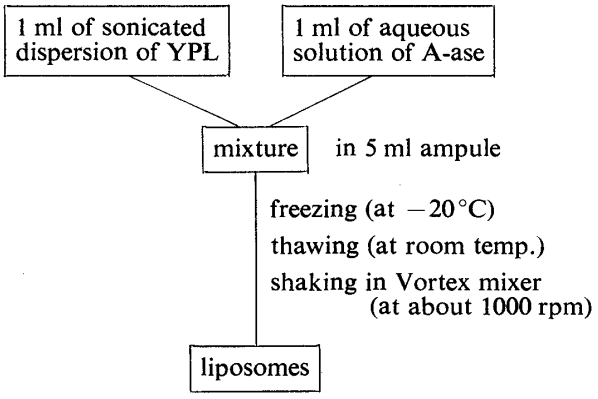

Fig. 1. Preparation of Liposomes by the Freeze-Thawing Method

ase determined by this method is referred to as *TA* hereafter.

The calibration curve showed a good linear relation between the amount of liberated ammonia and the A-ase concentration regardless of whether Triton X-100 was present or not.

Gel Filtration——Gel filtration was carried out on a column (1.6 cm diameter, 45 cm length) of Sephadex G-200 (Pharmacia Fine Chem. Sweden) equilibrated with Tris buffer at 5 °C. Fractions of 2 ml each were collected, and *FA* and *TA* in each fraction were determined by the methods described above.

Trypsin Treatment——Trypsin treatment was carried out according to the method reported by Fishman and Citri.[14] Trypsin solution (40 μg/ml in Tris buffer) was freshly prepared and mixed with liposomes containing A-ase. This mixture was incubated at 37 °C. The amounts of the liposomes and the trypsin solution were adjusted so that the concentrations of A-ase and trypsin in the reaction mixture were 2.5 IU/ml and 20 μg/ml, respectively. The reaction was terminated by the initiation of the determination of A-ase activity as described above.[14]

Results and Discussion

Electron Microscopic Observation

Figure 2 shows typical electron micrographs of negatively stained liposomes prepared by the FT and HY methods. In both cases, multilamellar structures were clearly observed and the shapes of liposomes were similar to each other. The range of particle size, D_e, of both liposomes was from about 0.1 to 1.0 μm in diameter. In order to clarify the inner structures of both liposomes, the particle size distributions were determined from the electron micrographs by measuring the longer diameters of about 200 liposomes. They showed a log normal distribution (Fig. 3), and the distribution functions were very similar to each other. The mean diameters calculated from the data in Fig. 3 were 259 and 249 nm for FT liposomes and HY liposomes, respectively.

As shown in Fig. 2, liposomes were composed of two different phases, that is, outer lamellar layers and an inner water phase which was located near the center of the particles and was surrounded by the outer lamellar layers. The largest diameter of this central water phase, D_i, was determined from micrographs of about 200 liposomes whose central water phases could be clearly observed. The distribution curves are also shown in Fig. 3; they have log normal distribution, and the mean diameters of the inner central water phases were 172 and 170 nm for FT liposomes and HY liposomes, respectively.

From the electron micrographs in Fig. 2, the thickness of one unit of lamellae (one unit is composed of one lipid bilayer and one water layer) could be estimated to be about 5 nm.

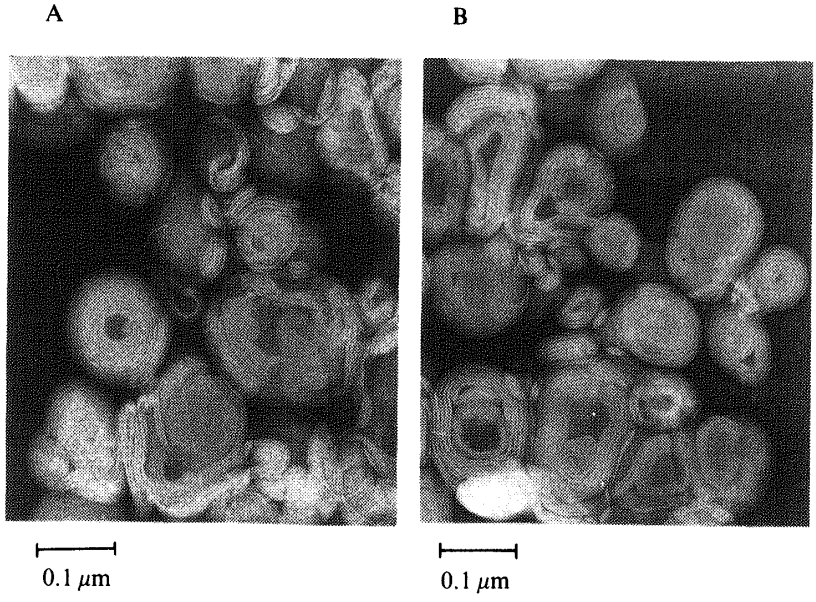

Fig. 2. Electron Micrographs of FT Liposomes (A) and HY Liposomes (B)

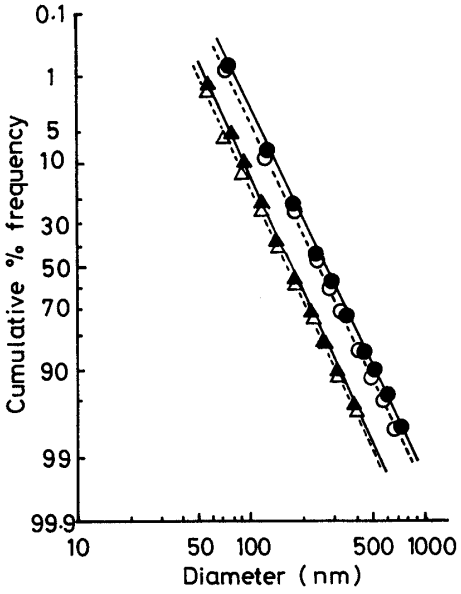

Fig. 3. Distribution of Particle Size of Liposomes (Circles) and the Diameter of the Central Water Phase (Triangles)

Symbols: closed, FT liposomes; open, HY liposomes.

TABLE I. Difference of Liposomal Structure Depending on Preparation Conditions

Sample	Shaking[a] conditions	Mean diameter[b] (nm) D_e	D_i	Thickness of lamellar layer $(D_e-D_i)/2$	Number of lipid bilayers
FT liposome	V	259	172	44	9
	US	90	35	28	6
HY liposome	V	249	170	40	8
	US	88	48	20	4

a) V, Vortex mixer (1000 rpm) for 20 min at room temperature; US, ultrasonic disintegrator (probe-type sonicator) for 2 min. *b*) D_e, the longer diameter of a liposome; D_i, the longer diameter of the central water phase in a liposome. Geometric mean diameter on a number basis was estimated from the log probability plot shown in Fig. 3.

Therefore, the average number of lipid bilayers of one liposomal particle could be estimated as $((D_e-D_i)/2)/5$; the calculated value was about 8 or 9 for each case.

In this paper, the shaking process after thawing was mainly performed in a Vortex mixer. This shaking process seemed to affect the size distribution, and thus shaking with a probe type sonicator for 2 min under nitrogen gas in an ice water bath was also tried for comparison. Table I shows that the shaking conditions had a marked effect on the structure. Sonication produced much smaller particles than the Vortex mixer irrespective of whether the FT method or HY method was used.

Evaluation of Encapsulation Efficiency (*EN*%)

1) Estimation from the Difference between *FA* and *TA*——*FA*** and ***TA*** were determined for the following four preparations: A) A-ase aqueous solution, B) simple mixture (a mixture of sonicated YPL suspension and A-ase solution which had not been frozen), C) FT liposomes and D) HY liposomes. The results are shown in Table II.

FA and *TA* values of the control A-ase aqueous solution were the same, indicating that the presence of Triton X-100 does not affect the assay of A-ase activity. The *FA* and *TA* values of the simple mixture were equal and were almost the same as those of the control solution. In the cases of FT and HY liposomes, *TA* values were almost the same as the control, whereas the *FA* values were much smaller than the *TA* values. These results suggested that the activity

TABLE II. A-ase Activity of the Four Preparations and $EN\%$ Values

Preparation	Activity (IU/ml)		$EN\%$		
	FA	TA	$EN(1)\%$	$EN(2)\%$	$EN(3)\%$
A-ase solution	10.83	10.83	0.0	0.0	0.0
Simple mixture[a)]	10.34	10.58	2.3	0.0	0.0
FT liposomes[a)]	5.49	10.99	50.0	56.0	53.6
HY liposomes[a)]	6.88	10.21	32.6	32.4	46.5

a) Each preparation contains 50 mg/ml of YPL as the lipid constituent.

of A-ase encapsulated in liposomes is masked by the liposomal membranes. Thus, $EN\%$ could be calculated using Eq. 1. The calculated values of $EN\%$ are shown in Table II as $EN(1)\%$ together with those obtained by other evaluation methods ($EN(2)\%$ or $EN(3)\%$).

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

It was clear that A-ase was scarcely encapsulated (only 2%) by simple mixing, but once the simple mixture was frozen and subsequently thawed, then A-ase was encapsulated in liposome to a considerable extent (50%). $EN(1)\%$ of HY liposomes was about 33%, and was lower than that of FT liposomes.

2) Gel Filtration Method——In order to confirm the encapsulation phenomena, gel filtration was performed on a Sephadex G-200 column for the above four samples. Fractions of 2 ml each were collected in a tube, and both FA and TA in each fraction were determined. The results are shown in Fig. 4. The control solution and the simple mixture gave only one peak at around 40 ml (Fig. 4A, 4B). However, in the cases of FT and HY liposomes, FA gave only one peak around 40 ml, but TA gave two peaks around 40 and 26 ml (Fig. 4C, 4D). The former peaks coincided with that of the control solution, and could be attributed to free A-ase. The latter peak corresponded to high molecular materials and could be attributed to liposomes. Blue dextran (molecular weight was about 2000000) showed almost the same elution volume as the latter peak (around 26 ml), as shown in Fig. 4A. $EN\%$ can be estimated from the gel filtration result by using Eq. 2 and is shown in Table II as $EN(2)\%$:

$$EN(2)\% = 100 \times A_{26}/(A_{26} + A_{40}) \quad (2)$$

where A_{26} and A_{40} mean the areas of the peaks at elution volumes of around 26 and 40 ml, respectively.

3) Trypsin Treatment——The extent of the resistance of liposomes to proteolysis was evaluated using trypsin. Trypsin was added to the above four preparations, then FA and TA were determined after appropriate incubation intervals. In addition, a liposomal preparation was lysed with Triton X-100, then trypsin was added and the mixture was incubated. The TA values were determined (these TA values are designated as TA_{ly}). FA, TA and TA_{ly} are shown in Fig. 5.

The TA, FA and TA_{ly} values of the control solution decreased rapidly to zero and they all showed very similar patterns (only that of TA is given in Fig. 5A). This means that A-ase was proteolyzed by trypsin. TA, FA and TA_{ly} of the simple mixture also decreased rapidly and showed values similar to those of the control solution (Fig. 5A). In the case of FT and HY liposomes, TA_{ly} was almost the same as TA of the control solution. TA also decreased rapidly; however, it did not reach zero but fell to a constant value. The extent of the decrease of TA, the difference of TA between the initial and equilibrium stages, was nearly equal to that of FA (Fig. 5C, 5D).

These results show that unencapsulated A-ase was inactivated rapidly by trypsin, but

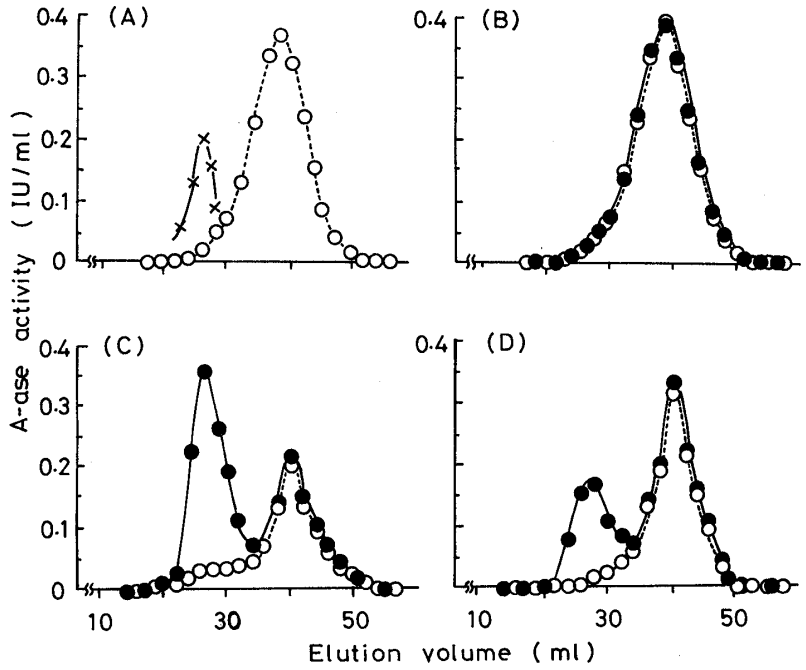

Fig. 4. Gel Filtration Patterns of A-ase in Aqueous Solution (A), Simple Mixture (B), FT Liposomes (C) and HY Liposomes (D)

---○---, *FA*; —●—, *TA*; —×—, blue dextran.

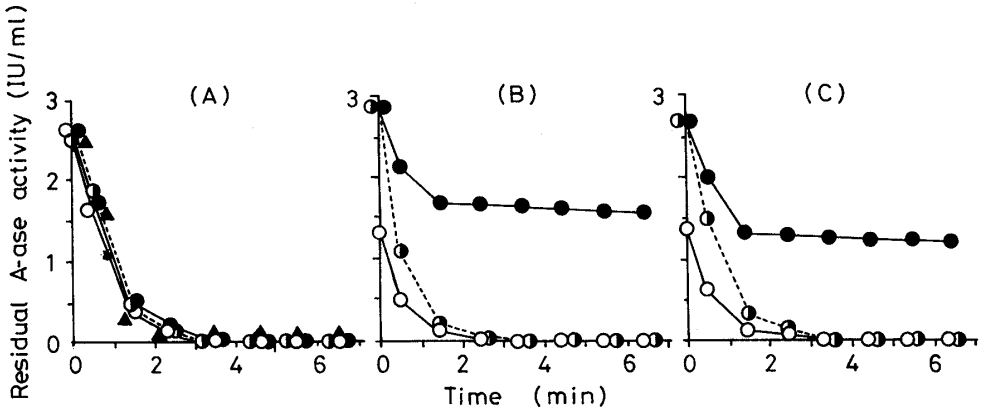

Fig. 5. Residual A-ase Activity of Simple Mixture (A), FT Liposomes (B) and HY Liposomes (C) after Trypsin Treatment

TA values of the control solution are also shown by closed triangles (---▲---) in A.
—○—, *FA*; —●—, *TA*; ---◑---, TA_{ly}.

encapsulated A-ase was completely resistant to proteolysis by trypsin. Thus, $EN\%$ can be estimated by using Eq. 3. The values were calculated for the four preparations and are shown in Table II as $EN(3)\%$.

$$EN(3)\% = 100 \times TA_{eq}/TA_{in} \tag{3}$$

Here TA_{eq} and TA_{in} mean the *TA* values at the equilibrium stage and the initial stage, respectively.

Resistance to Osmotic Pressure

Liposomes for use as a parenteral dosage form may be mixed with other drug solutions such as aqueous infusion or injection. The osmotic pressures of these solutions are usually

Table III. Comparison of Encapsulation Efficiency for A-ase of FT Liposomes with That of HY Liposomes

Preparation	*EN*%		
	EN(1)%	*EN*(2)%	*EN*(3)%
FT liposomes	56.0	55.8	53.6
	57.4	58.6	—
	50.0	—	45.3
	52.6	—	47.4
HY liposomes	32.1	32.4	—
	37.8	28.3	—
	54.0	—	46.5
	29.7	—	27.3

Table IV. Influence of the Stage of A-ase Addition during Liposome Preparation on *EN*%

Method	Stage of addition	*EN*(1)%
FT	1) Before freezing	52.1
	2) After thawing	25.1
	3) After agitating	0
HY	1) In aqueous phase	37.8
	2) After agitating	0

Each preparation finally contains 50 mg/ml of YPL and 10 IU/ml of A-ase.

adjusted to be isotonic. Thus, to determine the resistance of encapsulated A-ase to the osmotic pressure, 2 ml of FT liposomes was diluted with 6 ml of 1) Tris buffer, 2) 0.9% sodium chloride solution in Tris buffer or 3) 5% glucose solution in Tris buffer, and the time course of *EN*% was determined for 2 h. It was found that the *EN*(1)% maintained the initial level during 2 h in every solution. This shows that encapsulated A-ase does not readily leak from liposomes at the osmotic pressure of usual parenteral dosage forms.

Comparison of the FT Method and HY Method

To compare the reproducibility of the manufacturing procedure, several batches of liposomes were prepared by the FT method and HY method and *EN*% was determined by the three methods (Table III).

It was shown that *EN*% values agreed fairly well among the three evaluation methods, and FT liposomes gave higher average *EN*% with less variation among lots than HY liposomes. These results also indicate the superiority of the FT method to the HY method.

As the shapes, the particle size distributions and the multilamellar appearance of both liposomes were almost the same (Figs. 1 and 2), the difference in *EN*% is presumed to arise from the difference in the process of liposome formation. Thus, to investigate the effect of each process on *EN*%, the preparation was started from YPL and Tris buffer, and A-ase solution (20 IU/ml) was added at different stages as shown in Table IV.

The addition of A-ase after agitation had no effect on *EN*% in either case. This result and the resistance to leakage under the influence of osmotic pressure show that the migration of A-ase across the membrane hardly occurs after the liposomes are formed. The freezing process had the largest effect on *EN*% in the case of the FT method, and so the following explanation may be offered for the difference of *EN*%.

In the case of HY preparation, liposomes are presumed to be formed on the surface between the phospholipid film and continuous aqueous phase containing A-ase. Thus, the phospholipid layer nearest to the continuous phase is used first, then liposomes leave the surface region to enter the bulk solution, and a new phospholipid layer is exposed to the continuous phase. Liposomes are produced successively by contact of the newly exposed layer with the aqueous continuous phase. However, the concentration of A-ase in the continuous phase at this stage is presumably lower than that of the initial stage, because some of the A-ase has been concentrated in the initially formed liposomes. Thus, the later generated HY liposomes apparently give a lower $EN\%$, because the encapsulated amount of A-ase decreases with decrease of the A-ase concentration in the aqueous phase. The results of a detailed study will be presented in our next paper.

In the case of FT preparation, A-ase and phospholipid are dispersed beforehand and then frozen. During the freezing process, which has the largest effect on $EN\%$ (Table IV), the concentration of A-ase around each phospholipid can be regarded as uniform and the same as that of the beginning of HY preparation. Therefore, almost all FT liposomes seem to be prepared in an environment of higher A-ase concentration than in the case of HY liposomes, resulting in a higher $EN\%$.

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