Comparative Estimation of the Liposomal Content of Phosphatidylcholine/Triolein Emulsions Using Fluorescence Quenching and ^{1}H-NMR Techniques[1)]

Hiroaki KOMATSU,*,[a] Tetsurou HANDA,[b] and Koichiro MIYAJIMA[b]

Division of Drugs, Osaka Branch, National Institute of Health Sciences,[a] 1–1–43, Hoenzaka, Chuo-ku, Osaka 540, Japan and Faculty of Pharmaceutical Sciences, Kyoto University,[b] Yoshida-shimoadachi, Sakyo-ku, Kyoto 606–01, Japan. Received February 23, 1994; accepted April 15, 1994

The proportions of phosphatidylcholine (PC) molecules forming liposomes and the monolayer of emulsion particles in sonicated dimyristoyl or egg yolk PC/triolein dispersions (emulsions) were estimated by fluorescence quenching, using phosphatidylethanolamine with its hydrophilic group labelled with a fluorescent dansyl group, and by the ^{1}H-NMR signal shift of the choline-methyl proton of PC using a paramagnetic shift agent. From a comparison of the values estimated by the fluorescence and ^{1}H-NMR methods, it was concluded that the fluorescence method slightly underestimates the proportion of PC molecules in liposomes but can be effectively used to estimate the liposomal content of lipid dispersions due to the sensitivity and the relative simplicity of the basic methodology and instrumentation.

Keywords fat emulsion; liposome; fluorescence; NMR; egg yolk phosphatidylcholine; triolein

Fat emulsions composed of purified egg yolk phosphatidylcholine (eggPC) and soybean oil, with an average-particle diameter of about 250 nm,[2)] have been used as intravenous high-calory nutrient fluids and a drug carrier of alprostadil (prostaglandin E_1), dexamethasone palmitate and flurbiprofen axetil. For a long time, it was believed that they contained only oil-in-water (O/W) type-emulsion particles. Recently, however, on the basis of monolayer/bilayer equilibrium measurements on eggPC/triolein dispersions it has been suggested that liposomes coexist with emulsion particles (EMPs).[3)] In recent years, there has been a need for simple and accurate methods of determining the liposomal content of emulsions and electron microscopic and/or trapped volume techniques have been applied to emulsions of varying composition.[3,4)]

Fluorescence spectroscopy has been widely used to study the distribution of lipids on the surface of lipid molecular assemblies such as liposomes and micelles.[5)] A number of studies have also shown that NMR spectroscopy can be used to distinguish the inner from the outer surfaces of liposomal bilayer membranes.[6,7)] When liposomes and EMPs coexist in lipid dispersions (emulsions), phosphatidylcholine (PC) used as an emulsifier forms the inner and outer monolayers of liposomal bilayers and a monolayer of EMPs. If we can evaluate separately the fraction of PC in the respective monolayers, then the liposomal content in lipid dispersions can be estimated.

In the present study, the liposomal content of PC/triolein dispersions was estimated using fluorescence quenching and ^{1}H-NMR techniques and the respective advantages of both are discussed.

Experimental

Materials Dimyristoyl L-α-phosphatidylcholine (DMPC) (99%, Crystalline, P6392), L-α-phosphatidylcholine from egg yolk (eggPC) (99%, Type V-E, P5763) and L-α-lysophosphatidylcholine from egg yolk (egglysoPC) (99%, Type I, L4129) were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Fluorophore lipid, *N*-(5-dimethylaminonaphthalene-1-sulfonyl)-1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine triethylammonium salt (Dansyl-DHPE) was purchased from Molecular Probes, Inc., Eugene, OR, U.S.A. Triolein (glyceryltrioleate, TO) was obtained from Taiyo Chemical Co., Kyoto, Japan and purified by column chromatography using silica-gel (Wakogel C-200, Wako Pure Chemical Industries, Ltd., Osaka, Japan) with chloroform/ethanol (99/1, v/v) as eluent. The purity of TO thus obtained was over 99% as determined by thin-layer chromatography.

Praseodymium (III) nitrate hexahydrate and anhydrous copper (II) sulfate were supplied by Wako Pure Chemical Industries, Ltd. Praseodymium (III) nitrate hexahydrate was recrystallized twice from deuterium oxide solution and was then kept in a desiccator until required. Deuterium oxide (over 99.9% purity) was obtained from Aldrich Chemical Company, Inc., (Milwaukee, WIS, U.S.A.). Water was double-distilled using a quartz still.

Preparations DMPC, eggPC, Dansyl-DHPE and/or TO at various molar ratios were dissolved in chloroform. After evaporation of the solvent the mixtures were dried *in vacuo* for at least 12 h. Then 10 ml of buffer solution, containing 0.15 M NaCl and 0.01 M Tris–HCl in H_2O at pH 7.0 (because the pH values of commercially available intravenous high-calory nutrient fluids are about 7)[1)] for fluorescence and only 0.15 M NaCl in D_2O for NMR measurements, were added to the dried lipid mixture at 4 °C. The total concentration of lipids other than Dansyl-DHPE was kept at 0.2 mM for the fluorescence measurements and at 20 mM for the NMR measurements. In the former case, the concentration of Dansyl-DHPE was set at 5 mol% of DMPC or eggPC. The suspension was briefly vortexed, and then sonicated continuously for 40 min at an input power of 100 W under a stream of nitrogen gas. The temperature during vortexing and sonication was kept at about 65 °C. Details of the sonication time and the temperature during sonication have been discussed and presented elsewhere.[3)] The probe-type sonicator used was a UD-200 model from Tomy Seiko Co., Ltd., Tokyo, Japan. The lipid dispersions were centrifuged for 10 min at 2500 rpm to remove titanium dust.

5 ml of 10 mM egglysoPC micellar solution (critical micelle concentration of egglysoPC in water was 4.5×10^{-4} M)[8)] was prepared in a manner similar to the above method except that the preparation did not involve a sonication step.

Measurements Fluorescence Measurement: Fluorescence spectra were obtained at 25 °C using a Jasco FB-550 spectrofluorometer. The excitation wavelength was 335 nm. The fluorescence intensities were uncorrected.

Fluorescence titrations were performed by the stepwise addition of small aliquots of dense copper(II) sulfate solution (1 M) to the fluorescent lipid-containing solutions. Further details can be obtained from a previous paper.[9)] Corrections were made for the dilution of the fluorescent lipid, and did not exceed 5%.

NMR Measurement: ^{1}H-NMR spectra were recorded at 25 °C using

a Brucker AC-300 FT-NMR spectrometer operating at 300 MHz. 5 mm NMR glass tubes contained 1 ml samples.

Average Diameter and Diameter Distribution Measurements: Dynamic light-scattering (DLS) of samples was observed at 25 °C with a laser particle analyzer system LPA-3000/3100 (Photal, Otsuka Electronic Co., Ltd., Hirakata, Japan) at a scattering angle of 90°. The data were analyzed by the histogram method,[10] and then the average diameter and diameter distribution were evaluated.

Results and Discussion

Fluorescence Quenching Phosphatidylethanolamine, having a dansyl group attached to its hydrophilic portion (Dansyl-DHPE) was used as a fluorophore. Fluorescence spectra, having a peak at about 515 nm, decreased in intensity with increasing quencher Cu^{2+} concentration. Assuming that all liposomes are unilamellar and that there is an equal probability that Dansyl-DHPE is located in the outer and inner monolayers of liposomal bilayers and in the monolayers of EMPs, the ratio of the fluorescent intensities at 515 nm with (F) and without (F_0), a quencher is represented by Eq. 1.[11]

$$F/F_0=(F^{out}+F^{in})/(F_0^{out}+F_0^{in}) \quad (1)$$

where the superscripts "in" and "out" represent the inner monolayer of liposomal bilayers and the outer monolayers of liposomes and EMPs, respectively. As Cu^{2+} has a very low permeability as far as lipid membranes are concerned, the quencher cannot penetrate into the internal aqueous phase of liposomes. Therefore, the fluorescence intensity originating from the inner monolayer fluorophore of liposomes without a quencher is the same as that with quencher: $F_0^{in}=F^{in}$. If the molar fraction of Dansyl-DHPE in the outer monolayers of liposomal bilayers and EMPs is $R^{out}=F_0^{out}/(F_0^{out}+F_0^{in})$, Eq. 1 gives to

$$F/F_0=R^{out}(F^{out}/F_0^{out}-1)+1 \quad (2)$$

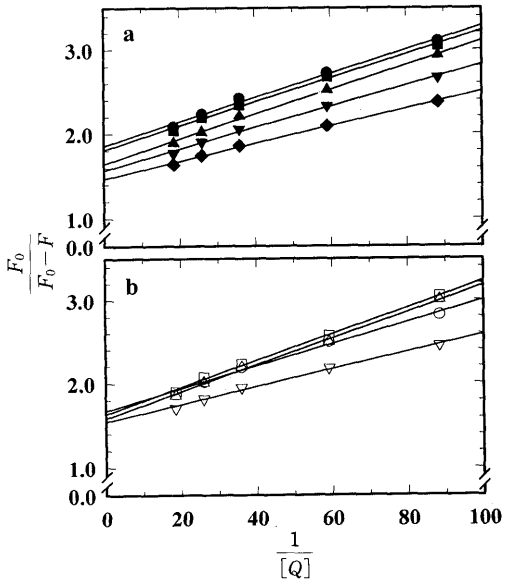

Fig. 1. $F_0/(F_0-F)$ *vs.* 1/[Q] Plots According to Eq. 4 for (a) DMPC/TO/Dansyl-DHPE and (b) eggPC/TO/Dansyl-DHPE Sonicated Dispersions

●, DMPC/Dansyl-DHPE = 200 μM/10 μM: ■, DMPC/TO/Dansyl-DHPE = 194 μM/6 μM/10 μM: ▲, 140 μM/60 μM/7 μM: ▼, 100 μM/100 μM/5 μM: ◆, 140 μM/60 μM/3 μM: ○, eggPC/Dansyl-DHPE = 200 μM/10 μM: □, eggPC/TO/Dansyl-DHPE = 140 μM/60 μM/7 μM: △, 100 μM/100 μM/5 μM: ▽, 140 μM/60 μM/3 μM.

The Stern–Volmer equation is applicable to quenching in the outer monolayer of liposomal bilayers and EMPs,

$$F_0^{out}/F^{out}=1+K_{sv}[Q] \quad (3)$$

where [Q] is the quencher concentration and K_{SV} the quenching constant. Substituting Eq. 3 in Eq. 2 gives the following equation,

$$F_0/(F_0-F)=1/(R^{out}\cdot K_{SV})\cdot 1/[Q]+1/R^{out} \quad (4)$$

Figure 1 shows $F_0/(F_0-F)$ *vs.* 1/[Q] plots for DMPC or eggPC/Dansyl-DHPE liposomes and DMPC or eggPC/TO/Dansyl-DHPE dispersions (emulsions), indicating that there is a linear relationship between $F_0/(F_0-F)$ and 1/[Q]. The R^{out} and K_{SV} values obtained from the slopes and intercepts with the ordinate axis are summarized in Table I.

R^{out} Values in Sonicated Liposomes Huang and Mason estimated the R^{out} value of the theoretically smallest eggPC liposomes as 0.68 (diameter: 19.8 nm, outer monolayer thickness: 2.1 nm, inner monolayer thickness: 1.6 nm).[12] Similar results were observed with sonicated DMPC liposomes. These R^{out} values are larger than ours, 0.60. The average diameter of our sonicated eggPC liposomes is 45.6±5.5 nm (S.D., $n=17$) and the size distribution exhibits two peaks, typically at 31.5 and 68.5 nm, accounting for 90 and 10%, respectively, in terms of a peak analysis by weight. This result is in line with Huang's finding that the size distribution of sonicated liposomes exhibits two peaks although the liposomes are relatively homodispersed.[13] An increase in the diameter of liposomes, namely, a decrease in the curvature, leads to a reduction in R^{out}. Assuming that the outer and inner monolayer thicknesses are 2.1 and 1.6 nm, respectively,[12] a diameter of 66.0 nm can be calculated from R^{out} value of 0.60. This diameter is considerably larger than the measured average value (45.6 nm). This implies the contamination of large liposomes and/or oligo-lamellar liposomes in the sonicated solution.

For the estimation of R^{out} we assumed that Dansyl-DHPE molecules are located in the outer and inner monolayers of liposomal membranes with the same probability. However, unbalanced lipid distributions in the outer and inner monolayers of liposomal bilayers have been reported.[14] The curvature of small liposomes, such as sonicated liposomes, is very high. This situation leads to close arrangements of the hydrophobic groups of lipids in the outer monolayer and of the hydrophilic groups in the inner monolayer.[14] The lipids distribute between the outer and inner monolayers in a manner which is dependent on lipid shape. Phosphatidylethanolamine is a corn-type lipid and the probability of its distribution in the inner monolayer of small liposomes is higher than in the outer monolayer. There is a high degree of probability that fluorophore Dansyl-DHPE is distributed in the inner monolayer and R^{out} values were estimated smaller than the corresponding PC values. This suggests that our fluorescent method slightly underestimates the R^{out} value.

R^{out} Values in Sonicated PCs/TO Dispersions Figure 2 shows the R^{out} value as a function of the TO molar fraction. The R^{out} value increases with an increase in the amount of TO. The increase in the amount of TO leads

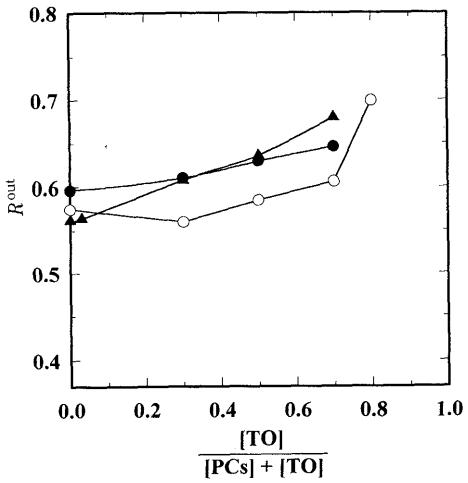

Fig. 2. Apparent Fractions (R^{out}) of PC Molecules in the Outer Monolayer of Liposomal Bilayers and EMPs with Varying Amounts of TO

DMPC/TO (●) and eggPC/TO (▲) evaluated by the fluorescence method, and eggPC/TO (○) estimated by the ^{1}H-NMR method in PCs/TO sonicated dispersions.

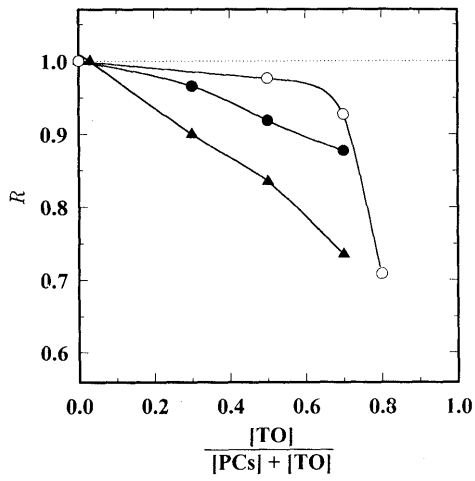

Fig. 3. Molar Fractions (R) of PC Molecules in Liposomes as a Function of TO Molar Fractions in the PCs/TO Sonicated Dispersions

DMPC/TO (●) and eggPC/TO (▲) evaluated by the fluorescence method, and eggPC/TO (○) estimated by the ^{1}H-NMR method.

TABLE I. Apparent Fractions (R^{out}) of PC Molecules in the Outer Monolayers of Liposomal Bilayers and EMPs with Varying Amounts of TO

DMPC/TO or eggPC/TO Molar ratios	R^{out}		
	Fluorescence method		^{1}H-NMR method
	DMPC/TO	eggPC/TO	eggPC/TO
10 /0	0.56 (1.1)	0.60 (1.3)	0.58
9.7/0.3	0.56 (1.2)		
7 /3	0.61 (1.1)	0.61 (1.0)	0.56
5 /5	0.64 (1.3)	0.63 (1.0)	0.59
3 /7	0.68 (1.4)	0.63 (1.5)	0.61
2 /8			0.70

The values in brackets are the K_{SV} constants.

to a decrease in the liposome content of the dispersions and is consistent with an equilibrium between emulsion and liposomal particles in PC/TO dispersions.[3]

The proportion of eggPC molecules forming liposomes in dispersions containing 60 μM eggPC and 140 μM TO is estimated as follows. The ratio of Dansyl-DHPE in the outer monolayers of liposomes and EMPs to that in the inner monolayer of liposomes, R^{in}, is 0.35 ($R^{in}=1-R^{out}$ and $R^{out}=0.65$ in Table I). It has been shown that liposomes consisting of 3 mol% TO and 97 mol% eggPC coexist with EMPs in equilibrium.[3] The value of 0.60 was used as the R^{out} value at the molar ratio of eggPC/TO = 9.7/0.3, that is, $R^{in}=0.40$. If the molar concentrations of PC molecules constituting the inner monolayer of liposomes, the outer monolayer of liposomes and the monolayer of EMPs in the lipid dispersions are a, b and c, respectively, the R^{in} of the liposomes ($R^{in}_{(L)}$) and the R^{in} of the liposomes and EMPs ($R^{in}_{(L+E)}$) can be described as follows;

$$R^{in}_{(L)}=a/(a+b) \quad \text{and} \quad R^{in}_{(L+E)}=a/(a+b+c) \tag{5}$$

Here, the solubility of PCs in the triolein core of EMPs is very small and can be ignored.[3] The molar fraction of PC molecules forming liposomes, R, may be derived from Eq. 5 to give Eq. 6.

$$R=R^{in}_{(L+E)}/R^{in}_{(L)}=(a+b)/(a+b+c) \tag{6}$$

As $R^{in}_{(L)}=0.40$ and $R^{in}_{(L+E)}=0.35$, a value of $R=0.88$ is obtained. Figure 3 depicts R values as a function of TO molar fractions.

^{1}H-NMR Spectra 1. R^{out} Values in Sonicated Liposomes Figure 4a shows the ^{1}H-NMR spectrum of sonicated eggPC-liposomes, including high resolution signals from the lipid acyl chains and choline head-groups, the latter being located at about 3.2 ppm.[15,16] On adjusting the concentration of Pr^{3+} in the external aqueous phase to 4 mM, the spectrum shown in Fig. 4b was obtained. The separate signals are now seen originating from the head-groups in the outer monolayer 'O' and in the inner monolayers 'I'. In the case of egglysoPC micelles, all head-groups face the aqueous phase. Addition of Pr^{3+} to the solution led to the down-field shift of all signals and this was accompanied by broadening of the signals (spectra not shown). The spectral separation of the liposomes is due to the down-field shift of signal 'O'.[17] Such shifts are caused by pseudecontact, dipolar interactions of Pr^{3+} with the choline groups in the outer monolayer.[18] The ratio of the areas of 'I' and 'O' signals is 0.74, and therefore, $R^{out}=1/(1+0.74)=0.58$.

Many workers have determined R^{out} values of 0.60—0.70 from the chemical shift, the broadening and the nuclear Overhauser effect of signals in ^{1}H-, ^{13}C- or ^{31}P-NMR spectroscopy.[7] Hutton *et al.* concluded that the metal interacts with all the PCs on the exposed bilayer surface[7] and an R^{out} ratio of 0.677 ± 0.010 could be obtained regardless of the nucleus species, position of the nucleus relative to the metal ion binding site or molar ratio of metal to PC, over three orders of magnitude.[7]

The R^{out} value of 0.677 is much higher than the value of 0.58 obtained in this study. This can be ascribed to contamination of large liposomes and/or oligo-lamellar liposomes in sonicated liposomes.

2. R^{out} Values in Sonicated PCs/TO Dispersions If liposomes and EMPs are mixed in dispersions, the ad-

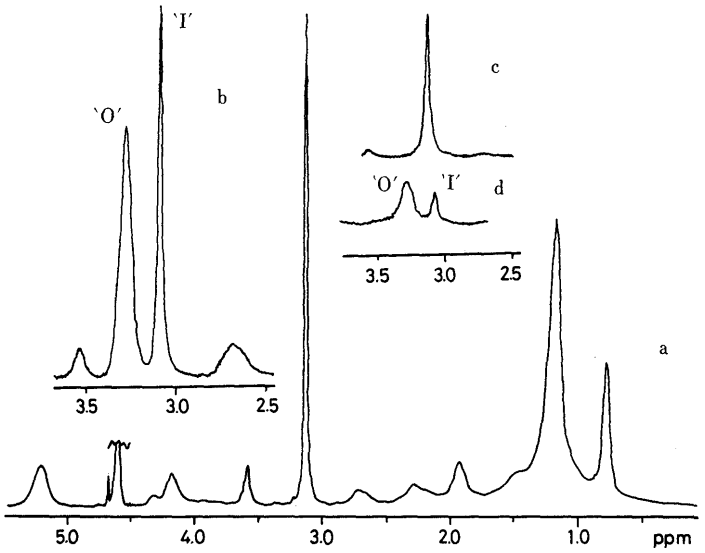

Fig. 4. ^{1}H-NMR Spectra of Sonicated eggPC and/or TO Dispersions, Including High Resolution Signals from the Choline Head-Groups

a and b, sonicated eggPC liposomes in the absence (a) and presence (b) of Pr^{3+} in the external aqueous phase at 4 mM; and c and d, sonicated eggPC/TO (8 mM eggPC/32 mM TO=2/8) dispersions in the absence (c) and presence (d) of Pr^{3+} in the external aqueous phase at 4 mM. A peak at about 3.2 ppm in the inserted figure a originates from the choline head-groups. The labels 'O' and 'I' indicate separate signals originating from the head-groups of eggPC molecules in the outer monolayer of liposomal bilayers and/or EMPs, and in the inner monolayer of liposomes, respectively.

dition of Pr^{3+} to the solution leads to the peak originating from the outer monolayers forming liposomal membranes and EMPs being shifted while the peak from the inner monolayer of liposomal membranes remains in its original position. Figures 4c and 4d show ^{1}H-NMR signals originating from the choline head-groups of PCs at a molar ratio of eggPC/TO=2/8 in the absence and presence of Pr^{3+} in the external-aqueous phase. The signal was split into two peaks by the addition of Pr^{3+}. This signal splitting indicates the existence of an internal aqueous phase and a proportion of the PCs are presumed to face this internal phase. An R^{out} value of 0.70 was obtained from the ratio of the areas. Table I summarizes the R^{out} values obtained from ^{1}H-NMR spectroscopy. Figure 2 also illustrates plots of the R^{out} values as a function of the TO molar fractions. The R^{out} values are relatively small in comparison with those obtained by the fluorescence method and increase with an increase in the proportion of TO.

The molar fraction, R, of eggPC molecules forming liposomes in eggPC/TO dispersions (emulsions) is estimated from the NMR measurements using the same calculation involving Eqs. 5 and 6 in the fluorescence method (Fig. 3).

The proportion of liposomes in a dispersion containing 8 mM eggPC and 32 mM TO (2:8 molar ratio) may be estimated as follows. As the R value is 0.71 from the above calculation, the concentration of eggPC forming liposomes is 5.66 mM and, therefore, the EMPs consist of 2.34 mM eggPC and 32 mM TO. The DLS measurement indicates that the size distribution has two peaks at 42.6 ± 2.0 nm and 175.6 ± 81.0 nm (S.D., $n=10$) with peak analysis by weight as a typical parameter. It has been suggested that EMPs below about 50 nm in diameter can not be formed in eggPC/TO.[3] So, we can assume that liposomes have an average diameter of 42.6 nm the corresponding figure for EMPs is 175.6 nm. Assuming that the volume per eggPC molecule is 1.253 nm^3 and that the outer and inner monolayer thicknesses are 2.1 nm and 1.6 nm, respectively,[12] the number of eggPC molecules per liposome particle is 1.41×10^4. In addition, assuming that the outer monolayer thickness of EMPs is 2.1 nm, the number of eggPC molecules per EMP is 1.586×10^4. Therefore, the dispersed solution contains 96% liposomes and 4% EMPs, in terms of the number of colloidal particles.

Thus, the molar fraction of PC molecules in liposomes and/or the fraction of liposomes in PCs/TO dispersed solutions can be estimated.

Methodological Comparison of Fluorescence Quenching and NMR Spectroscopy Chiba and Tada applied NMR spectroscopy to studies of the relationship between the stability of PC/decane/water W/O/W emulsions and the PC distribution in the aqueous phases inside and outside an EMP.[19] It has been shown that the ^{31}P-NMR spectra of emulsions, in the presence of Pr^{3+}, allow the quantitative measurement of PCs exposed to the internal and external aqueous phases. However, the NMR method has a number of disadvantages, including the large samples size required, the long time needed for measurement in some cases and the expense of the necessary equipment.

Fluorescence spectroscopy has been widely used to study the structure and dynamics of model lipid membranes and biological membranes. In the present study, while it appears that the fluorescence method slightly underestimates R^{out} values, its sensitivity and relative simplicity of the methodology and instrumentation indicate that it is suitable for estimating the liposomal content of lipid dispersions. Therefore, the fluorescence method can be used to qualitatively distinguish between PC molecules in the inner and outer monolayers of PC liposomal bilayers.

Acknowledgements A part of this work was supported by Grant

HS-3-2-3 from the Japan Health Sciences Foundation. The authors thank Mr. Kazumasa Nishijima for technical assistance. We are grateful to Dr. Satoshi Okada, National Institute of Health Sciences, Japan, for helpful comments and discussions and would like to express our appreciation to Taiyo Chemical Co. for providing TO. We also thank Dr. Malcolm A. Moore for comments on the manuscript.

References

1) A part of this work was presented at the 109th Annual Meeting of the Pharmaceutical Society of Japan, Nagoya, April 1989.
2) H. Komatsu, A. Kitajima, S. Okada, Abstracts of Papers, the 114th Annual Meeting of Pharmaceutical Society of Japan, Tokyo, March 1994.
3) T. Handa, H. Saito, K. Miyajima, *Biochemistry*, **29**, 2884 (1990); *idem*, *Biophys. J.*, **64**, 1760 (1993).
4) T. Handa, Y. Asai, K. Miyajima, Y. Kawashima, M. Kayano, K. Ida, T. Ikeuchi, *J. Colloid Interface Sci.*, **143**, 205 (1991); T. Handa, Y. Asai, H. Komatsu, K. Miyajima, *ibid.*, **153**, 303 (1992).
5) R. P. Haugland, "Handbook of Fluorescent Probes and Research Chemicals," 5th ed., ed. by K. D. Larison, Molecular Probes, Inc., Eugene, 1992, p. 235.
6) M. S. Fernandez, J. Cerbon, *Biochim. Biophys. Acta*, **298**, 8 (1973); U. F. Bystrov, Y. E. Shapiro, A. V. Viktorov, L. I. Borsukov, L. D. Bergelson, *Febs. Lett.*, **25**, 337 (1972).
7) W. C. Hutton, P. L. Yeagle, R. B. Martin, *Chem. Phys. Lipids*, **19**, 255 (1977).
8) E. Hamori, A. M. Michaels, *Biochim. Biophys. Acta*, **231**, 496 (1971).
9) M. Nakagaki, H. Komatsu, T. Handa, *Chem. Pharm. Bull.*, **34**, 4479 (1986).
10) E. Gulari, E. Gulari, Y. Tsunashima, B. Chu, *J. Chem. Phys.*, **70**, 3965 (1979).
11) T. Handa, K. Matsuzaki, M. Nakagaki, *J. Colloid Interface Sci.*, **116**, 50 (1987).
12) C. Huang, J. T. Mason, *Proc. Natl. Acad. Sci. U.S.A.*, **75**, 308 (1978).
13) C. Huang, *Biochemistry*, **8**, 344 (1969).
14) C. G. Brouillette, J. P. Segrest, T. C. Ng., J. L. Jones, *Biochemistry*, **21**, 4569 (1982); L. O. Sillerud, R. E. Barnett, *ibid.*, **21**, 1756 (1982).
15) V. F. Bystrov, N. I. Dubrovina, L. J. Barsukov, L. D. Bergelson, *Chem. Phys. Lipids*, **6**, 343 (1971); J. A. Berden, R. W. Barker, G. K. Radda, *Biochim. Biophys. Acta*, **375**, 186 (1975); H. Hauser, M. C. Phillips, B. A. Levine, R. J. P. Williams, *Eur. J. Biochem.*, **58**, 133 (1975); S. B. Andrews, J. W. Faller, J. M. Gilliam, R. J. Barnett, *Proc. Natl. Acad. Sci. U.S.A.*, **70**, 1814 (1973).
16) D. M. Michaelson, A. F. Horwitz, M. P. Kein, *Biochemistry*, **12**, 2637 (1973).
17) R. A. Hunt, L. R. H. Tipping, *J. Inorg. Biochem.*, **12**, 17 (1980).
18) L. D. Bergelson, "Methods in Membrane Biology," Vol. 9, ed. by E. D. Korn, Plenum Press, New York, 1977, p. 275.
19) K. Chiba, M. Tada, *Agric. Biol. Chem.*, **54**, 907 (1990).