Effects of Incorporation of Various Amphiphiles into Recipient Liposome Membranes on Inter-Membrane Protein Transfer

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To obtain information about the factors governing spontaneous inter-membrane protein transfer, we examined the effects of incorporation of various amphiphilic compounds in dimyristoylphosphatidylcholine (DMPC) liposomes on protein transfer from influenza virus-infected cells to the liposomes, and analyzed the physical properties of these liposome membranes. The incorporation of amphiphilic compounds, negatively charged dicetylphosphate (DCP), dipalmitoylphosphatidylserine (DPPS) or positively charged dimethyldipalmitoylammonium (DMDPA), into DMPC liposomal membranes enhanced protein transfer. The liposomes containing DCP, DPPS or DMDPA were unaffected by osmotic shock caused by external addition of glucose, suggesting a decrease in lipid packing in the liposomal membranes. Furthermore, calorimetric study of these liposomes showed that a phase separation occurred partially in the liposomal membranes. Accordingly, the membranes of DMPC liposomes containing DCP, DPPS and DMDPA should be distorted due to the coexistence of two phases, gel and liquid crystalline, in the membranes. Consequently, the membrane distortion could be responsible for the enhancement effects of the amphiphiles on the inter-membrane protein transfer from influenza virus-infected cells to the liposomes.

Key words protein transfer; liposomes; phase separation; virosome

Recently, several groups have reported the interesting phenomenon that membrane proteins of various cells transfer spontaneously to liposomal membranes on incubation without detergents or organic solvents.[2—16)] Huestis and coworkers reported the inter-membrane transfer of anion transporter band 3 from erythrocytes to liposome membranes.[5,6,9,15)] Their finding is particularly interesting, because band 3, which transferred to liposomal membranes on incubation without any additions, is a protein that spans the plasma membrane multiple times.[17)] Furthermore, the transferred band 3 protein retained its original activity and native orientation after transfer to liposomal membranes.[9)] Thus inter-membrane protein transfer seems to be an ideal method for membrane protein reconstitution. In addition, Huestis's group reported that inter-membrane protein transfer is affected by relative differences in membrane fluidity of the donor and acceptor.[5)] On the other hand, Sunamoto and his coworkers found that the incorporation of the artificial lipid 1,2-dimyristoylamido-1,2-deoxyphosphatidylcholine (DDPC) into phosphatidylcholine (PC) liposomal membranes enhanced spontaneous transfer of various membrane proteins, such as band 3,[10)] taste receptor proteins,[11)] platelet membrane protein[12)] and erythrocyte glycophorin[13)] to PC liposomes. We have studied the inter-membrane transfer of viral membrane protein from influenza virus-infected cells to liposomal membranes for development of the artificial-membrane vaccine virosome, in which antigenic viral proteins are reconstituted.[14,16)] We have found that incorporation of the negatively charged amphiphilic molecule dicetylphosphate (DCP) into liposomal membranes enhances the inter-membrane transfer of protein from virus-infected cells to dimylistoylphosphatidylcholine (DMPC) liposomes.[16)] In this study, to obtain more information for efficient preparation of virosome, we examined the effects of incorporation of various amphiphilic molecules into recipient membranes on protein transfer from influenza virus-infected cells to DMPC liposomes, and analyzed the changes in the physical properties of DMPC membranes in the presence of these components.

Materials and Methods

Materials DMPC and DPPC were obtained from Nichiyu Liposome Co. (Tokyo, Japan). DCP, bovine brain phosphatidylserine (bbPS) and dipalmitoylphosphatidylserine (DPPS) were purchased from Sigma Chemical Co. (St. Louis, U.S.A.). Dimethyldipalmitoylammonium (DMDPA) bromide was obtained from Tokyo Kasei Kogyo Co. (Tokyo, Japan). The chemical structures of the lipids and the amphiphiles are shown in Fig. 1. Other materials and reagents were of the highest grade commercially available.

Cell Culture and Infection with Influenza Virus CV-1 cells from monkey kidney were used as the host of influenza virus NWS strain (H1N1). Procedures for cell culture and infection with virus were as described previously.[14,16)] The expression of viral membrane proteins was confirmed by the appearance of the viral glycoprotein hemagglutinin on the cell surface.[16)]

Preparation of Liposomes Liposomes were prepared by reverse phase evaporation[18)] in phosphate-buffered saline (PBS) consisting of 137 mM NaCl, 2.68 mM KCl, 8.45 mM Na_2HPO_4 and 1.47 mM KH_2PO_4. For preparation of liposomes containing DMDPA, buffer consisting of 10 mM HEPES–NaOH (pH 7.4) and 266 mM glucose (HEPES–glucose buffer) was used instead of PBS, because salt caused the liposomes to aggregate. The liposomes were extruded over ten times through polycarbonate membrane filters to obtain those with a diameter of about 200 nm. Their diameter was determined with a laser particle analyzer (LPA 3000/3100, Otsuka Electronics Co., Osaka, Japan). The amount of phospholipids in liposome suspensions was determined as phosphorus by the method of Ames.[19)]

Incubation of Liposomes with Virus-Infected Cells The incubation procedure was as described previously.[14,16)] Cells grown to confluence in 35-mm dishes were washed three times with buffer. Then 1 ml of 0.1 mMPi liposome suspension described above was added to the dishes, and they were incubated for 1 h at 37 °C without shaking. The length of incubation was decided by our previous experience, *i.e.*, the amount of transferred protein after 30 min incubation was almost the same after 1 h (unpublished data), and a number of cells were removed from the dish by incubation longer than 1 h. The liposome suspension was then recovered and centrifuged at $2000\times\boldsymbol{g}$ for 1 min to remove cells coexisting in the suspension. The liposomes were recovered almost completely (unpublished data). The protein content of the recovered liposome suspension was estimated by the method of Lowry *et al.*[20)] using bovine serum albumin as a standard.

Measurement of the Response of Liposomes to Osmotic Shock The response of liposomes to osmotic shock was followed spectroscopically by change in absorbance at 450 nm as described by Shibata *et al.*[21)] Osmotic shock was induced by rapid addition of 40 μl of various concentrations of glucose into 1.96 ml of 1.5 mMPi liposomal suspension at 37 °C, with stir-

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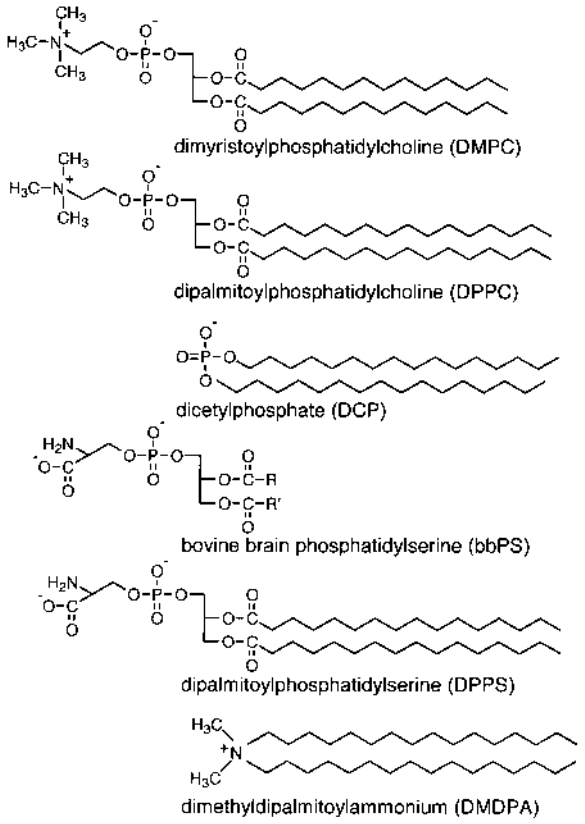

Fig. 1. Diagram of Chemical Structures of All Lipids and Amphiphiles Used in This Study

ring, and the change in turbidity due to shrinkage of liposomes was monitored with a spectrophotometer (UV-160A, Shimadzu Co., Kyoto, Japan). The initial water permeability velocity (v) across the liposomal membranes induced by the hypertonic stress of addition of a high concentration of glucose was determined by the equation.[21,22]

$$v=(\mathrm{d}A/\mathrm{d}t)_{t=0}/A_{t=0} \quad (1)$$

where $A_{t=0}$ is the absorbance of the liposome suspension at the time of injection of glucose ($t=0$). Since v is proportional to the initial rate of change of liposome volume, $(\mathrm{d}V/\mathrm{d}t)_{t=0}$, the relationship is as follows;[21]

$$v=k(\mathrm{d}V/\mathrm{d}t)_{t=0}=kP_{\mathrm{w}}S\boldsymbol{R}T\Delta C_{\mathrm{glc}} \quad (2)$$

where k is a constant, P_{w} is the water permeability coefficient, S is the surface area of the membranes, $\boldsymbol{R}$ is the gas constant, T is the absolute temperature, and ΔC_{glc} is the difference between the glucose concentrations outside and inside the liposomal membranes. The change in v was dependent on P_{w} (Eq. 2), because the other parameters were constant under the present experimental conditions. Therefore, a decrease in v can be regarded as a decrease in water permeability across the liposomal membranes. The trace was taken into a computer with a scanner, and the initial velocity of turbidity change was determined by the least-squares method.

Measurement of the Phase Transition Temperature of Liposomal Membranes The phase transition temperatures of various liposomal membranes were measured by differential scanning calorimetry (DSC). Liposome samples for DSC measurement were prepared as described above with deionized distilled water instead of the buffer. Samples of 30 μl of 40 mM Pi liposome suspension were placed into an aluminum sample cups and sealed. A reference cup contained 30 μl of deionized distilled water. The cups were placed in a DSC cell compartment (Daini-Seikosha, Model SSC-560) and cooled to about 5 °C with ice. The temperature was raised at a rate of 1 °C/min to 120 °C and measurements were started from 7—10 °C.

Results

Effect of Various Amphiphilic Components in Liposomes on Inter-Membrane Protein Transfer First we examined the effects of incorporations of various amphiphilic components into DMPC liposomal membranes on protein transfer from influenza virus-infected cells to liposomes. In the absence of added amphiphilic compounds, the amount of protein transferred from virus-infected cells to liposomes containing only DMPC on incubation for 1 h at 37 °C was 21.3($\pm$1.5) μg/ml (n=5). As shown in Fig. 2, inter-membrane protein transfer was enhanced significantly by the presence of 30 mol% DCP (for structure, see Fig. 1) in DMPC liposomal membranes as we reported previously.[16] However, the incorporation of DPPC having long saturated hydrocarbon chains of the same length as those of DCP into DMPC liposomes did not enhance protein transfer. Addition of the negatively charged amphiphile bbPS to DMPC liposomal membranes also did not affect protein transfer. On the contrary, the incorporation of DPPS (having two saturated acyl chains) into DMPC liposomes enhanced inter-membrane protein transfer. Alkylammonium DMDPA (for structure, see Fig. 1), which, unlike DCP, is positively charged, significantly enhanced inter-membrane protein transfer, though its effect was less than that of DCP (Fig. 2).

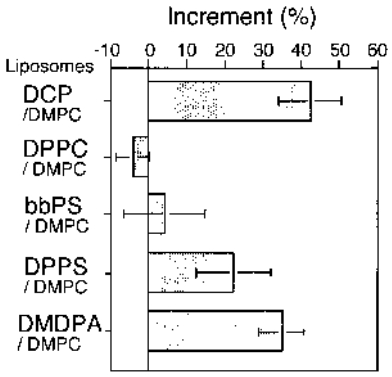

Fig. 2. Increments in Protein Transfer from Influenza Virus-Infected Cells to Various Liposomes as Percentages of That to Liposomes with DMPC Only

The contents of amphiphilic compounds in DMPC liposomes were 30 mol%. The transfer of protein to control liposomes with DMPC only was 21.3$\pm$1.5 μg/ml (n=5). Percentage increments were calculated from differences in the amount of protein transferred to liposomes with DMPC only and those with DMPC and amphiphiles. DMDPA/DMPC liposomes were suspended in HEPES–glucose buffer, and other liposomes in PBS. Other experimental conditions were as described in Materials and Methods.

Table 1. Initial Velocities of the Increase in Turbidity of DMPC Liposomes Containing Various Amphiphilic Compounds Induced by the Addition of Hypertonic Glucose Solution

	Liposomes					
	DMPC	DCP/ DMPC	DPPC/ DMPC	bbPS/ DMPC	DPPS/ DMPC	DMDPA/ DMPC
v ($\pm$S.D.)	0.039 ($\pm$0.0015)	ND	0.018 ($\pm$0.0049)	0.0045 ($\pm$0.0015)	ND	ND

Values are averages for 3 or more experiments ($\pm$standard deviations). The contents of amphiphilic compounds in DMPC liposomes were 30 mol%. ND, not detected.

Responses of Liposomal Membrane to Osmotic Shock To determine the membrane state of the various liposomes shown in Fig. 2, we examined their responses to osmotic shock by the addition of glucose solution at 37 °C (Table 1). In general, liposomes, in which the state of lipids is uniform,

should shrink immediately with the permeation of water from inside to outside due to the difference in osmotic pressure caused by glucose addition.[22] In this study, the turbidity of DMPC liposome suspension increased immediately by addition of glucose solution at 37 °C, indicating liposome shrinkage with water permeation, and the initial velocity of increase in turbidity, *v*, depended on the concentration of glucose (data not shown). Accordingly, DMPC liposomes are able to function as osmometers, and the state of lipids in DMPC liposomes should be a uniform liquid crystalline state at 37 °C. Similarly, DMPC liposomes containing DPPC (DPPC/DMPC liposomes) and bbPS (bbPS/DMPC liposomes) showed a response to added glucose (Table 1). As the *v* value of bbPS/DMPC liposomes was very low, their membranes have very low water permeability like membrane-stabilized liposomes containing the acidic phospholipid bovine heart cardiolipin[21] and egg phosphatidic acid.[22] However, the turbidities of suspensions of DCP/DMPC liposomes and DMPC liposomes containing DMDPA or DPPS (referred to as DMDPA/DMPC liposomes and DPPS/DMPC liposomes, respectively) did not change on addition of even the highest concentration of glucose used (final concentration 20 mM). Thus these liposomes (DCP/DMPC liposomes, DMDPA/DMPC liposomes and DPPS/DMPC liposomes) were concluded not to function as osmometers, *i.e.*, glucose molecules readily pass through their membranes from the outside. Therefore, the lipid packing of these liposomal membranes should be weakened significantly by the presence of the amphiphiles at 37 °C.

Analysis of the Physical State of Liposomal Membranes by DSC To obtain more information about the state of DMPC membranes containing amphiphiles such as DCP, DMDPA or DPPS, we analyzed their physical states by DSC. As shown in Fig. 3, DMPC liposomes showed a sharp endothermic peak indicating a gel-to-liquid crystalline phase transition at about 24 °C, as reported previously.[23]

In contrast to the DSC profile for DPPC/DMPC or DSPC/DPPC mixed vesicles, where phospholipids are known to be miscible, the peak position of DMPC liposomes changed only slightly on incorporation of DCP, and the peak became broader and smaller with increasing DCP content. Moreover, when the DCP contents were 20 and 30 mol%, another small, broader peak appeared at higher temperature, which was assigned to DCP, together with the peak of DMPC, and above 50 mol% DCP, the position of the peak assigned to DCP was almost the same as that of DCP alone, despite the coexistence of DMPC. On the other hand, up to 20 mol% DMDPA in DMDPA/DMPC liposomes shifted the position of the endothermic peak significantly to a higher temperature like DPPC, but with above 30 mol% DMDPA, the peak position was the same as that with DMDPA alone. These results suggest that when the contents of the DMPC liposome membranes are 30 mol% of the amphiphiles DCP or DMDPA, the contents do not become dispersed and mixed uniformly with the DMPC molecules; thus phase separation is possibly occurring in the membranes.

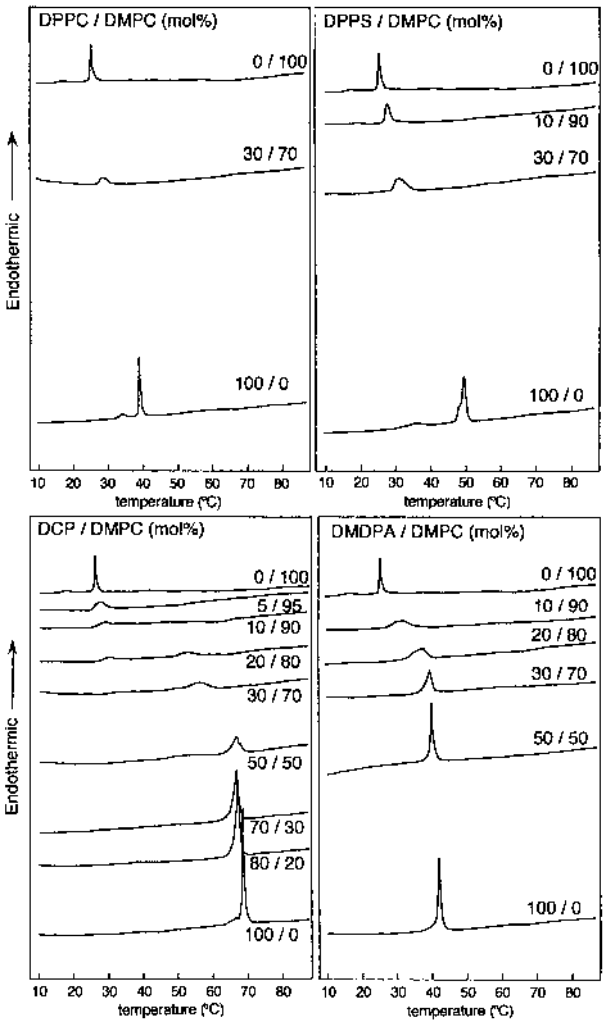

Fig. 3. DSC Curves of DMPC Liposomes Containing Various Amounts of the Amphiphilic Compounds, DPPC, DPPS, DCP and DMDPA

The ratios of the contents of amphiphilic compounds to that of DMPC are indicated beside the DSC curves.

Discussion

In this study, we examined the effects of the amphiphilic compounds DCP, DPPC, bbPS, DPPS and DMDPA on inter-membrane protein transfer, and analyzed the physical state of liposomal membranes containing these compounds. Consistent with our previous report,[16] the incorporation of DCP into DMPC liposomes increased protein transfer (Fig. 2). However, DPPC did not enhance protein transfer at 37 °C (Fig. 2). Therefore, the length of the hydrocarbon chains and the lowering of membrane fluidity by the presence of such long alkyl chains do not seem to be responsible for regulation and enhancement of protein transfer by DCP. This is consistent with our previous report [16] that membrane fluidity is not related to the amount of protein transfer. Furthermore, we found that the presence of DMDPA in DMPC liposomes enhanced inter-membrane protein transfer (Fig. 2). It is noteworthy that DMDPA had the same effect as DCP, although, unlike DCP, it has a positive charge. On the other hand, bbPS in DMPC liposomes did not increase protein transfer, whereas DPPS did (Fig. 2). Thus although bbPS and DPPS have the same head group and a negative charge, their effects on inter-membrane protein transfer were different. From these results, we conclude that the electrostatic conditions on the surface of liposomal membranes are not important for inter-membrane protein transfer, and the reasons for the effects of DCP, DMDPA and DPPS at 37 °C. This conclusion is

consistent with a report by Waters *et al*.[15)]

Next, to clarify the relationship between protein transfer and the physical state of liposome membranes, we compared the responses to osmotic shock at 37 °C (Table 1) and carried out a calorimetric study (Fig. 3) to compare DCP/DMPC liposomes, DMDPA/DMPC liposomes and DPPS/DMPC liposomes with liposomes containing DMPC only. DCP/DMPC liposomes, DMDPA/DMPC liposomes and DPPS/DMPC liposomes did not show any response to osmotic shock at 37 °C (Table 1), suggesting that the barrier of DMPC liposomal membranes against glucose was lost on incorporation of DCP, DMDPA or DPPS, and the state of these membranes was not uniform, *i.e*., that gel and the liquid crystalline states coexist at 37 °C. Furthermore, phase separation was suggested to occur in membranes containing DCP and DMDPA (Fig. 3). But DMPC and DCP or DMDPA are probably only partially mixed, because the peak of endothermic gel-to-liquid crystalline phase transition of DMPC in the liposome membranes became small and broad in the presence of DCP and DMDPA (Fig. 3). In addition, as DMPC liposomes containing 30 mol% DPPS showed an endothermic peak at near 37 °C (Fig. 3), that is, as the phase transition temperature should be nearly 37 °C, the gel and the liquid crystalline states would coexist at 37 °C.

These results suggested that at 37 °C DMPC was in the liquid crystalline state, and DCP and DMDPA were in the gel state in DCP/DMPC liposomes and DMDPA/DMPC liposomes, and that the membranes of DPPS/DMPC liposomes should be in both the gel and liquid crystalline states. Accordingly, the membranes of DCP/DMPC liposomes, DMDPA/DMPC liposomes and DPPS/DMPC liposomes should be distorted due to the coexistence of gel and liquid crystalline states in the membrane. In addition, the distorted membranes due to phase separation might be stabilized by insertion of transferred protein into the interface between the two phases to decrease the interfacial energy.

Previously, Waters reported that sodium cholate, well known as an ionic detergent, enhanced inter-membrane protein transfer of band 3 protein from erythrocytes to DMPC liposomes.[15)] They suggested that disruption of lipid packing by the detergent was responsible for that effect. In their experiment, since they added the detergent into the mixture of recipient liposomes and donor erythrocytes, interactions of lipid–lipid and lipid–protein would have been weakened due to the existence of cholate in both membranes. However, this was not the case in our experiments; *i.e*., the presence of DCP, DMDPA or DPPS in DMPC liposomes would not affect the conditions of the donor, and not facilitate the release of protein from cell membranes, because translocation of the amphiphiles such as DCP from liposomal membranes to interact with cells like cholate should be difficult due to their high energy.

In this study, we examined the effects of incorporation of various amphiphiles into DMPC liposomes on inter-membrane protein transfer from influenza virus-infected cells to liposomes. We found that several amphiphiles DCP, DMDPA and DPPS, which distort the liposome membranes, enhanced protein transfer and concluded that membrane distortion due to coexistence of the gel and liquid crystalline states is responsible for the effects of the amphiphiles. Further studies are in progress to clarify the mechanism of the enhancing effect of membrane distortion on inter-membrane protein transfer.

References and Notes

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2) Roseman M. A., Holloway P. W., Calabro M. A., Thompson T. E., *J. Biol. Chem*., **252**, 4842—4849 (1977).
3) Leto T. L., Holloway P. W., *J. Biol. Chem*., **254**, 5015—5019 (1979).
4) Leto T. L., Roseman M. A., Holloway P. W., *Biochemistry*, **19**, 1911—1916 (1980).
5) Cook S. L., Bouma S. R., Huestis W. H., *Biochemistry*, **19**, 4601—4607 (1980).
6) Newton A. C., Cook S. L., Huestis W. H., *Biochemistry*, **22**, 6110—6117 (1983).
7) Greenhut S. F., Roseman M. A., *J. Biol. Chem*., **260**, 5883—5886 (1985).
8) Greenhut S. F., Bourgeois V. R., Roseman M. A., *J. Biol. Chem*., **261**, 3670—3675 (1986).
9) Huestis W. H., Newton A. C., *J. Biol. Chem*., **261**, 16274—16278 (1986).
10) Sunamoto J., Goto M., Akiyoshi K., *Chem. Lett*., **1999**, 1249—1252.
11) Nakamura M., Tsujii K., Katsuragi Y., Kurihara K., Sunamoto J., *Biochem. Biophys. Res. Commun*., **201**, 415—422 (1994).
12) Okumura Y., Ishitobi M., Sobel M., Akiyoshi K., Sunamoto J., *Biochim. Biophys. Acta*, **1194**, 335—340 (1994).
13) Suzuki K., Okumura Y., Sato T., Sunamoto J., *Proc. Jpn. Acad*., **71B**, 93—97 (1995).
14) Kogure K., Itoh M., Hayashi K., Ueno M., *Biol. Pharm. Bull*., **18**, 1168—1170 (1995).
15) Waters S. I., Sen R., Brunauer L. S., Huestis W. H., *Biochemistry*, **35**, 4002—4008 (1996).
16) Kogure K., Nakamura C., Okuda O., Hayashi K., Ueno M., *Biochim. Biophys. Acta*, **1329**, 174—182 (1997).
17) Vince J. W., Reithmeier R. A. F., *Cell. Mol. Biol*., **42**, 1041—1051 (1996).
18) Szoka F., Olson F., Heath T., Vail W., Meyhew E., Papahadjopoulos D., *Biochim. Biophys. Acta*, **601**, 559—571 (1980).
19) Ames B. N., *Meth. Enzymol*., **8**, 115—118 (1966).
20) Lowry O. H., Rosebrough N. J., Farr A. L., Randall R. J., *J. Biol. Chem*., **193**, 265—275 (1951).
21) Shibata A., Ikawa K., Shimooka T., Terada H., *Biochim. Biophys. Acta*, **1192**, 71—78 (1994).
22) Blok M. C., van Deenen L. L. M., De Gier J., *Biochim. Biophys. Acta*, **433**, 1—12 (1976).
23) Blume A., *Biochemistry*, **22**, 5436—5442 (1983).