Liposomal Membranes. III. Permeation of Pyrene-labeled Lecithin into Matrix of Liposomal Bilayers

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The permeation of a novel pyrene-labeled phosphatidylcholine, DPDL (1,2-bis[10-(1-pyrenyl)decanoyl]-*sn*-glycero-3-phosphatidylcholine), into the dipalmitoyl and egg phosphatidylcholine liposomes was studied. DPDL can adopt a sandwich conformation at the ground state, which comes in the fluorescence excimer emission. The fluorescent probe undergoes the initial rapid adsorption onto the liposome surfaces along with the instantaneous fluorescence enhancement followed by the relatively slow permeation into and diffusion in the liposomal bilayers. Through monitoring the relative quantum efficiency, I_e/I_m (see text), during the permeation, suggestive information about the bilayer structure is obtained at least around the probe. The initial binding to and the subsequent diffusion in the membrane occur mostly as the monomeric form of the probe. At lower temperature without cholesterol the bilayer structure seems to be a *lipid-separated* conformation (Fig. 1 (c)) and above the chain-melting transition the bilayer seems to exist as a *lipid-intimate* conformation (Fig. 1 (b)). These results completely coincide with those obtained in our previous study on the codispersion of the probe with phospholipids (J. Sunamoto, *et al.*, *J. Am. Chem. Soc.*, **102**, 1146 (1980)).

In a preceding paper we described syntheses of fluorescent probes, DPDL (1,2-bis[10-(1-pyrenyl)-decanoyl]-*sn*-glycero-3-phosphatidylcholine) and EP (ethyl 10-(1-pyrenyl)decanoate), and their fluorescence characteristics in the liposomal bilayers.[1] DPDL carries a pyrene moiety at the end of each fatty acyl chain of the lipid and can adopt a sandwich configuration of the pyrene moieties in the ground state, which fluorescence the excimer emission from the intramolecular ground state dimer.[1,2] When DPDL was codispersed with phosphatidylcholines, it could be intercalated in good order in the liposomal bilayers and gave meaningful informations about the structure of liposomal bilayers. (1) The relative quantum efficiency (the intensity ratio of the excimer emission at 480 nm to the monomer emission at 378 nm from DPDL, I_e/I_m) was significantly affected by the bilayer structure. (2) The addition of cholesterol into liposomes made the relative quantum efficiency larger. (3) Increasing temperature also caused an increase in the I_e/I_m ratio of DPDL in the egg phosphatidylcholine (softer) bilayers. The value always exceeded that in the dipalmitoyl phosphatidylcholine (more rigid) bilayers. (4) The observed I_e/I_m-temperature profile was thermotropically reversible. From these previous results, we proposed a conformational equilibrium of the liposomal bilayer structure as a function of temperature and as affected by cholesterol (Fig. 1). In order for the liposomal membranes to hold the observed bilayer thickness (46.5—46.8 Å)[3] with fully extended hydrocarbon chains (28.3 Å), the lipid molecule must be either the tilted conformation by about 30—35 degrees with respect to the bilayer plane (structure (a) in Fig. 1) or a shrunk one with hydrocarbon chains perpendicular to the bilayer plane ((b) or (c) in Fig. 1) at least below the chain melting transition. The codispersion studies using the probe DPDL suggested that below the chain melting transition without cholesterol the lipid bilayer structure is very probably the *lipid-separated* conformation (c), which comes in the predominant monomer emission. Above the chain melting transition or in the presence of cholesterol, on the other hand, the probability for the liposomal bilayers to be the *lipid-intimate* conformation (b) increases, which produces more excimer and results in the larger I_e/I_m ratio.

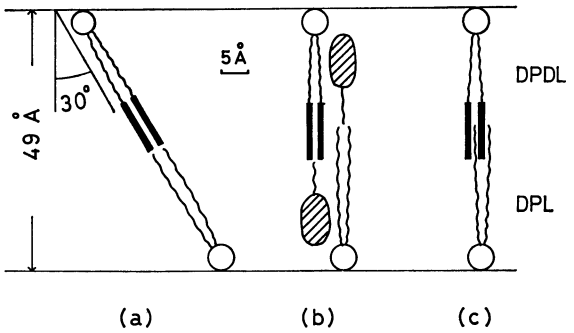

Fig. 1. Schematic representation of the structure of liposomal bilayers. The blocked group represents the pyrene moiety of DPDL. The hatched group represents the steroid nucleus of cholesterol. (a) Fully extended and tilted conformation. (b) The shrunk structure with the lipid-intimate conformation. (c) The shrunk structure with the lipid-separated conformation.

The interaction of the hydrophobic probes with liposomal membranes occurs in two successive kinetic phases; the initial rapid adsorption of probes onto the liposome surface and the second slow diffusion in the liposomal bilayer or transport across the bilayers.[4,5] In this work, we studied the permeation of DPDL or EP into the liposomal membranes and the diffusion of the probes in the bilayers in slow kinetic phase. Through the dynamic studies on the permeation and diffusion of the probes in the liposomal bilayers, the lipid conformation around the probe molecule will be discussed.

Experimental

Materials. Synthetic dipalmitoyl-DL-α-phosphatidylcholine was purchased from Sigma Chemical Company, St. Louis, Mo. and purified prior to use. Egg yolk lecithin was isolated and purified from egg yolk.[1,6,7] 1,2-Bis[10-(1-pyrenyl)decanoyl]-*sn*-glycero-3-phosphatidylcholine (DPDL) and ethyl 10-(1-pyrenyl) decanoate (EP) were the same as those used in the previous investigation.[1] Prior to use, the

purity of all the lecithins employed was inspected by TLC on silica-gel plate (Tokyo Kasei Spot Film S-160) with the solvent chloroform–methanol–water (65 : 25 : 4 by vol.).[8] All the detections by iodine staining, UV light irradiation, and Dragendorff's reagent showed only one spot of the corresponding lecithin.

Preparation of Liposomes. Multicompartment liposomes were prepared by the injection technique according to the method previously described.[1] Single compartment liposomes were formed by sonication technique and isolated by the gel-filtration using Sepharose 4B column.[7] Formations of single and multicompartment liposomes were respectively visualized by negatively staining with 2% aqueous potassium tungstophosphate on a JEOL JEM-100 V electron microscope. Phospholipid concentrations were determined as inorganic phosphate by the Allen's methods.[9]

Fluorescence Measurements. All the fluorescence measurements were made under atmospheric conditions on a Hitachi 650-10S fluorospectrometer exciting at 342 nm with the use of slits of 2 nm for excitation side and 10 nm for emission side, respectively. Under the conditions, good reproducibilities were ensured. Since the fluorescence intensity is proportional to the quantum yield when the exciting light intensity and concentration of absorbing molecule are kept constant, the fluorescence intensities for the monomer (378 nm) and the excimer (480 nm) from the pyrene moiety were determined.

For the permeation of fluorescent probes into bilayers of single-walled liposomes, an appropriate amount of ethanolic solution of the probes was injected using a microsyringe into the liposome solutions preincubated for 10 min at a given temperature. The content of ethanol in liposome solutions was depressed less than 8% (v/v). All the runs were duplicated.

Results and Discussion

Permeations of EP and DPDL. When EP was dispersed into the aqueous buffered solution, it showed the strongly quenched emission of fluorescence excimer but no monomer emission, and formation of small micelles or aggregates was electronmicrographically visualized. On the other hand, when EP was added into the liposome solutions, significant emissions of both fluorescence monomer and excimer were observed. The monomer emission as a function of time, then, gradually increased with a concomitant decrease of the excimer emission with an isolampsic (isoemissive) point at 442 nm (Fig. 2). When the fluorescent probe interacts with the liposomal bilayers, two distinct kinetic phases are observable.[4,5] They are a rapid initial adsorption of the probe onto the outer surface of liposomes and a much slower lateral diffusion in the outer half of bilayers[10] or transport across the inner layer of liposomal membranes.[4a] The instantaneous increase of fluorescence emission and the subsequent slow enhancement of the monomer emission suggest that aggregates or micelles of EP bind onto the liposome surface and subsequently the probe is intercalated in bilayers. Several typical examples of the change in individual values of I_e and I_m as a function of time are illustrated in Fig. 3. During 10 or 20 min after injection of the probe, both values usually level off and the system attains equilibrium. When EP was codispersed with phospholipids, no excimer emission was observed at

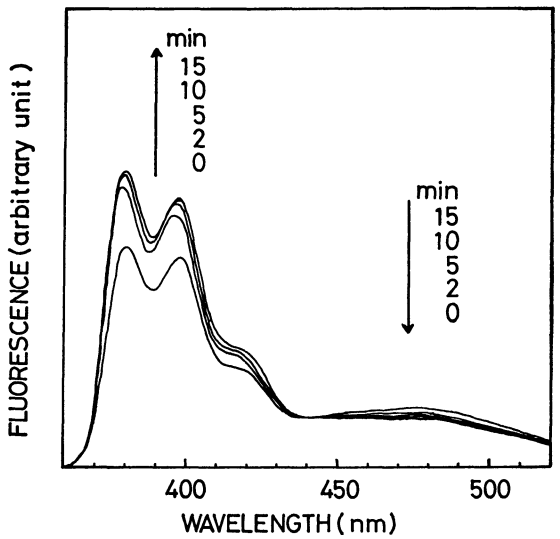

Fig. 2. Fluorescence spectra of EP as a function of time when EP was added into the multicompartment liposome solutions containing 7.84×10^{-6} M of dipalmitoyl lecithin in 0.028 M acetate buffer with 0.10 M NaCl at 25.0 °C. The final concentration of EP was 7.75×10^{-8} M in cuvette.

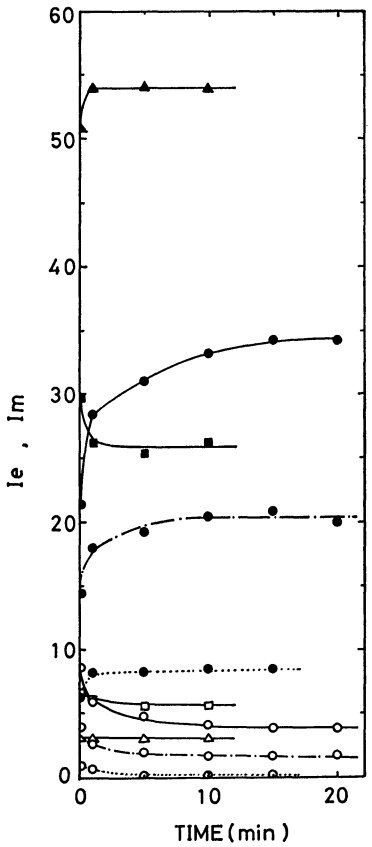

Fig. 3. Fluorescence intensities of excimer (I_e) and monomer (I_m) emissions from EP as a function of time. Conditions should be referred to Table 1. Run numbers are the same as those used in Table 1. Closed symbols are monomer emission, while open symbols are excimer emission: —●— and —○— are run 1; -·-●-·- and -·-○-·-, run 2; ···●··· and ···○···, run 3; —■— and —□—, run 4; and —▲— and —△—, run 6, respectively.

TABLE 1. RELATIVE QUANTUM EFFICIENCIES, I_e/I_m, IN EQUILIBRIUM UNDER VARIOUS CONDITIONS

Run	[Probe]/10^8M	Incubation temp °C	$\left(\frac{I_e}{I_m}\right)_{equilibrium}$	Run	[Probe]/10^8M	Incubation temp °C	$\left(\frac{I_e}{I_m}\right)_{equilibrium}$
(A): EP				(B): DPDL			
	Single-walled liposome of dipalmitoyl lecithin without cholesterol[a]				Single-walled liposome of dipalmitoyl lecithin without cholesterol[a]		
1	7.75	25	0.10	27	7.65	25	0.75
2	3.90	25	0.08	28	3.90	25	0.64
3	1.95	25	0.04	29	1.95	25	0.54
4	7.75	46	0.21	30	7.65	46	0.91
5	3.90	46	0.13	31	3.90	46	0.86
	Single-walled liposome of dipalmitoyl lecithin with cholesterol[b]			32	1.95	46	0.80
6	7.75	25	0.06		Single-walled liposome of dipalmitoyl lecithin with cholesterol[b]		
7	3.90	25	0.04	33	7.65	25	0.61
8	1.95	25	0.06	34	3.90	25	0.55
9	7.75	46	0.06	35	1.95	25	0.55
10	3.90	46	0.05	36	7.65	46	1.04
11	1.95	46	0.04	37	3.90	46	0.96
	Multilamellar liposome of dipalmitoyl lecithin without cholesterol[c]			38	1.95	46	0.69
12	7.75	25	0.17		Multilamellar liposome of dipalmitoyl lecithin without cholesterol[c]		
13	3.90	25	0.12	39	7.65	25	0.44
14	7.75	45	0.31	40	3.90	25	0.47
15	3.90	45	0.17	41	7.65	45	0.78
	Multilamellar liposome of dipalmitoyl lecithin with cholesterol[d]			42	3.90	45	0.72
16	7.75	25	0.13		Multilamellar liposome of dipalmitoyl lecithin with cholesterol[d]		
17	3.90	25	0.09	43	7.65	25	0.49
18	7.75	45	0.20	44	3.90	25	0.56
19	3.90	45	0.14	45	7.65	45	0.82
	Single-walled liposome of egg lecithin without cholesterol[e]			46	3.90	45	0.69
20	7.75	25	0.07		Single-walled liposome of egg lecithin without cholesterol[e]		
21	3.90	25	0.06	47	7.65	25	0.69
22	7.75	45	0.05	48	3.90	25	0.69
23	3.90	45	0.12	49	7.65	45	0.96
	Multilamellar liposome of egg lecithin without cholesterol[f]			50	3.90	45	1.14
24	7.75	25	0.27		Multilamellar liposome of egg lecithin without cholesterol[f]		
25	7.75	45	0.40	51	7.65	25	1.10
26	3.90	45	0.30	52	3.90	25	0.92
				53	7.65	45	1.21
				54	3.90	45	0.82

a) [dipalmitoyl lecithin]$=7.80\times10^{-6}$ M. b) [dipalmitoyl lecithin]$=7.80\times10^{-6}$ M and [cholesterol]$=3.86\times10^{-6}$ M.
c) [dipalmitoyl lecithin]$=7.84\times10^{-6}$ M. d) [dipalmitoyl lecithin]$=7.73\times10^{-6}$ M and [cholesterol]$=4.30\times10^{-6}$ M.
e) [egg lecithin]$=4.18\times10^{-6}$ M. f) [egg lecithin]$=4.02\times10^{-6}$ M.

all.[1] In the present case, however, as reflected on the relative quantum efficiency, I_e/I_m (Fig. 4), a small extent of the excimer emission still remained even in the slow second kinetic phase. This means that a portion of EP molecules may permeate into bilayers as the small aggregates. The concentration of EP injected, temperature, existence of cholesterol, sort of employed lecithin, and state of liposomes, that is, multi or single compartment liposomes all the factors have an influence on the I_e/I_m ratio (Fig. 4 and Table 1). With an increase in the initial concentration of EP added, the I_e/I_m ratio increased. Mostly in the multilamellar liposomes than in the single-walled liposomes, in single-walled liposomes of dipalmitoyl lecithin than in that of egg lecithin, and at higher incubation temperatures than the chain melting transition temperature of the adopted lecithin, the larger I_e/I_m values are obtained (though with several exceptions).

Differently from the case of EP, when DPDL was dispersed into an aqueous buffered solution containing no liposomes, the bilayer structure was obviously visualized by electronmicrography. And the fluorescence emission from DPDL was completely quenched. On the other hand, when DPDL was injected from the exterior of liposomes, the fluorescence quantum yield of the probe significantly increased. The initial and

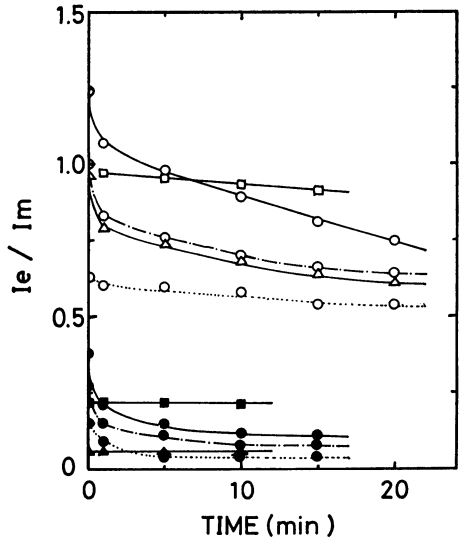

Fig. 4. The relative quantum efficiencies I_e/I_m of DPDL and EP in the single-walled liposomes of dipalmitoyl lecithin as a function of time. Open symbols are for DPDL, while closed symbols for EP: —●—, run 1; -·-●-·-, run 2; ···●···, run 3; —■—, run 4; —▲—, run 6; —○—, run 27; -·-○-·-, run 28; ···○···, run 29; —□—, run 30; and —△—, run 33, respectively. Run numbers should be referred to Table 1.

instantaneous enhancement of fluorescence from DPDL is interpreted in terms of the rapid uptake of the disorganized probe by liposomes and the consequent change in polarity of the microenvironment around the probe.[4a] Similarly to the case of EP, after the instantaneous enhancement of fluorescence emission, the monomer emission gradually increased with a concomitant decrease of the excimer emission (Fig. 5). The I_e/I_m values for DPDL as a function of time are

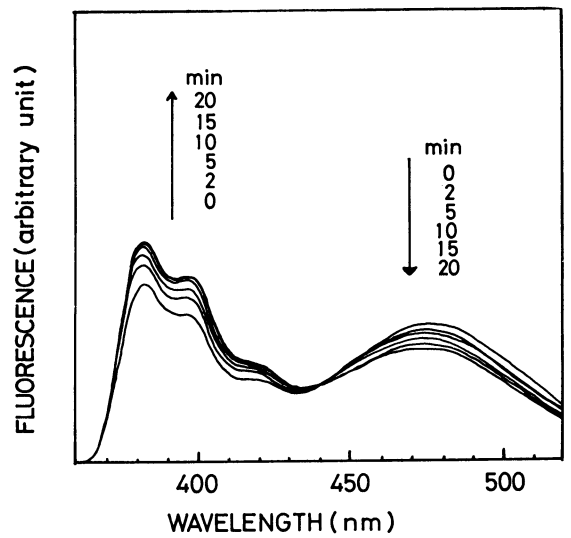

Fig. 5. Fluorescence spectra of DPDL as a function of time in the single-walled liposome of dipalmitoyl lecithin at 25 °C. DPDL (7.65×10^{-8} M) was added from exterior of the liposomes (7.80×10^{-6} M) in 0.028 M aqueous actate buffered solution containing 0.10 M NaCl.

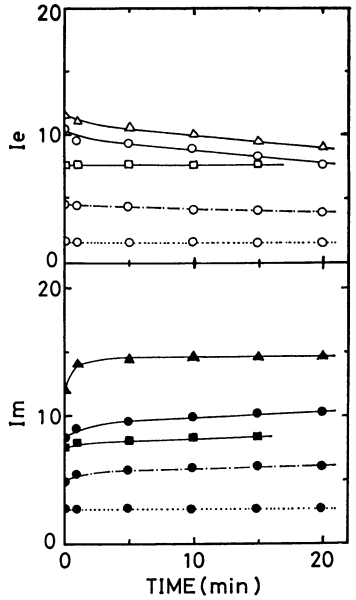

Fig. 6. Fluorescence intensities of excimer emission (I_e, open symbols) and monomer emission (I_m, closed symbols) from DPDL as a function of time in the single-walled liposome of dipalmitoyl lecithin: —●— and —○— are run 27; -·-●-·- and -·-○-·-, run 28; ···●··· and ···○···, run 29; —■— and —□—, run 30; and —▲— and —△, run 33, respectively. Run numbers should be referred to Table 1.

seen in Fig. 4 along with those for EP. The I_e/I_m ratios for DPDL are always larger than those for EP (Fig. 4 and Table 1). Comparing with the case of codispersion of DPDL with lecithins, more DPDL seem to be intramolecular excimer or the ground-state dimer during the permeation. It is true also for EP. Differently from the case of codispersion experiments, in the present study even EP shows the excimer formation. The lecithin analogue probe, DPDL, seems to diffuse more slowly in liposomal bilayers comparing with the smaller and neutral probe, EP. Fluorescence intensities of excimer and monomer emissions from DPDL as a function of time are plotted, respectively, in Fig. 6. With the increase in the initial concentration of DPDL injected and with raising temperature, the I_e/I_m ratio for DPDL increased (Table 1). However, effects of state of liposomes on the I_e/I_m ratio of DPDL are rather complicated. Contrary to what would be expected, the I_e/I_m ratios for DPDL in dipalmitoyl lecithin liposomes were always higher in single compartment liposomes than in multicompartment liposomes irrespective of the incubation temperature and the presence of cholesterol. However, for egg lecithin liposomes the values were larger in multilamellar liposomes than in single-walled liposomes, which is similarly seen in the case of EP. Since the apparent surface area of single compartment liposomes is larger than that of multicompartment liposomes if the lecithin concentration in solutions were the same, the apparent concentration of the probe bound to the liposome surface should be higher in the latter liposomes than in the former ones.[11] This will lead to the higher I_e/I_m ratio. However, this is not the

case for DPDL in the dipalmitoyl lecithin liposomes.

Lenz and his coworkers previously stated that the uptake of DPH (1,6-diphenyl-1,3,5-hexatriene) by multicompartment liposomes is a more complex process than that by single-walled liposomes; the fluorescence intensity of DPH increases in stepwise fashion in multilamellar liposomes.[5] Differently from the case of DPH, permeations of DPDL and EP both were relatively simple even into multilamellar liposomes. The rate of fluorescence enhancement in the slower kinetic phase must concern with the diffusion velocity or mobility of the probes in bilayers.[4,5,10] It is, therefore, reasonable that the I_e/I_m values of the probes attain equilibrium rather rapidly in fluid liposomal bilayers containing cholesterol or being incubated at higher temperatures. From facts that trans-membrane movements of phosphatidylcholine,[12] cholesterol,[13] and lysophosphatidylcholine[14] are extremely slow, the probes (DPDL and EP) are expected to be locating mostly in the outer half of bilayers,[4] especially when they are penetrated from the exterior of liposomes at low temperature.

Conformation of DPDL in Matrix of Liposomal Bilayers. Clearly from our previous study,[1] the I_e/I_m ratio for DPDL intercalated in liposomal bilayers will give a noteworthy information about the structure of bilayers at least around the probe. The high I_e/I_m ratio suggests a conformation by which the probability to take ground-state dimer increases (structure (b) in Fig. 1). Alternately, if the low I_e/I_m ratio were obtained, it suggests that DPDL should take the lipid-separated conformation in bilayers (structure (c) in Fig. 1). The relative quantum efficiencies obtained by the present permeation technique show substantially similar tendency to those obtained by the codispersion experiment.[1] That is, in more fluid liposomes such as egg lecithin liposomes than dipalmitoyl lecithin liposomes or liposomes incubated at higher temperatures than the phase transition temperature, DPDL shows larger values of I_e/I_m in equilibrium. For DPDL, there is no large difference also in the actual value of I_e/I_m ratio between the two distinct experiments. The I_e/I_m values from DPDL lie in a range of 0.5—1.0 and never exceed the value of 1.7 obtained for DPDL in ethanol.[1] Hence, it is reasonable to consider that DPDL permeates into and laterally diffuses in liposomal bilayers mostly with a lipid-separated conformation especially at low incubation temperature in the dipalmitoyl lecithin bilayers. Increasing the mobility of lipid and probe molecules in the hydrophobic domain of bilayers enhances the probability to take more lipid-intimate conformation.

Effect of cholesterol is completely different between the codispersion[1] and permeation studies. In our previous codispersion experiments,[1] addition of cholesterol abolished the phase transition of dipalmitoyl lecithin liposomes and increased the fluidity of membranes. In the present studies, for both probes (EP and DPDL) and for both lecithin liposomes (egg lecithin and dipalmitoyl lecithin), except the permeation of DPDL into multilamellar liposomes of dipalmitoyl lecithin, the addition of cholesterol diminished the I_e/I_m value during the permeation of the probes. This means that effect of cholesterol on the diffusion of the probe is different between the two cases, the codispersion and the permeation.

References

1) J. Sunamoto, T. Nomura, H. Okamoto, and H. Kondo, *J. Am. Chem. Soc.*, **102**, 1146 (1980).
2) a) D. R. G. Brimage and R. S. Davidson, *J. Chem. Soc., Chem. Commun.*, **1971**, 1385; b) R. S. Davidson and K. R. Trethewey, *ibid.*, **1976**, 827; c) R. S. Davidson and T. D. Whelan, *ibid.*, **1977**, 361; d) M. Goldenberg, J. Emert, and H. Morawetz, *J. Am. Chem. Soc.*, **100**, 7171 (1978).
3) M. J. Janiak, D. M. Small, and G. G. Shipley, *Biochemistry*, **15**, 4575 (1976).
4) a) T. Y. Tsong, *Biochemistry*, **14**, 5409 (1975); b) **14**, 5415 (1975).
5) B. R. Lentz, Y. Barenholz, and T. E. Thompson, *Biochemistry*, **15**, 4521 (1976).
6) J. S. Chadha, *Chem. Phys. Lipids*, **4**, 104 (1970).
7) J. Sunamoto, H. Kondo, and A. Yoshimatsu, *Biochim. Biophys. Acta*, **510**, 52 (1978).
8) W. S. Singleton, M. S. Grey, M. L. Brown, and J. L. White, *J. Am. Oil Chem. Soc.*, **42**, 53 (1965).
9) R. J. L. Allen, *Biochem. J.*, **34**, 858 (1940).
10) L. A. Sklar, B. S. Hudson, and R. D. Simoni, *Biochemistry*, **16**, 819 (1977).
11) M. A. Schwartz and H. M. McConnell, *Biochemistry*, **17**, 837 (1978).
12) L. W. Johnson, M. E. Hughes, and D. B. Zilversmit, *Biochim. Biophys. Acta*, **375**, 176 (1975).
13) M. Pozansky and Y. Lange, *Nature*, **259**, 420 (1976).
14) B. de Kruyff, A. M. H. P. van den Besselaar, and L. L. M. van Deenen, *Biochim. Biophys. Acta*, **465**, 443 (1977).