Liposomal Membranes. IV. Fusion of Liposomal Membranes Induced by Several Lipophilic Agents

Junzo SUNAMOTO,* Tomiko HAMADA, and Hiroki MURASE

Department of Industrial Chemistry, Faculty of Engineering, Nagasaki University, Nagasaki 852

(Received February 27, 1980)

It was found that several lipophilic agents, such as *cis*-, *trans*-ethyl decaprenoate and their structural analogues, significantly induce the liposome-liposome fusion when incubated together a set of two different liposomes, the *A*-liposome containing a marker and the *B*-liposome involving a lipophilic agent. The rate and extent of fusion were followed spectrophotometrically by monitoring the leakage of an amphiphilic marker, Bromothymol Blue, from the fused liposomes. Fusion was also visualized directly by electronmicrographic technique. When only *A*-liposome was incubated or when a blank liposome containing no lipophilic agents was added to the *A*-liposome after the preincubation, neither the fusion nor the marker release was observed at least in periods of several hours. Also, neither fusion nor the marker release was observed when either *A*- or *B*-liposome or both were multicompartment liposome. Removing cholesterol from liposome significantly depressed the fusion. The effect of cholesterol was more prominent on the *A*-liposome side. *trans*-Ethyl decaprenoate was more effective about three-fold to induce the fusion than the *cis*-isomer. Interestingly, the effect was parallel to the antihypertensive activity of the agent.

The transformation of liposomal membranes has generated much interest because of its possible analogy to biomembrane fusion and phagocytosis of importance in both membrane biology[1] and drug delivery system.[2] Taupin and McConnell were the first to suggest that small single compartment liposomes transform to larger multicompartment liposomes involving liposome-liposome fusion process in periods of several hours when incubated at appropriate temperatures.[3] Thereafter, the Ca(II) ion,[4-6] fatty acids,[7-9] and surface-active drugs[10,11] have been found to accelerate the fusion process of liposomes. In the case of Ca(II)-induced fusion, the key event leading to membrane fusion is the isothermal phase separation induced by Ca(II) ion.[5] The fatty acid-induced fusion also required lateral separation of a fatty acid rich phase.[7] Alamethicin, which is an extracellular cyclic-polypeptide antibiotic and a kind of surface active agents, stimulated fusion of liposomes followed by the preliminary interaction with the polar choline headgroup of the lecithins.[10] These earlier investigations suggest to us that perturbation of the membrane surface by the electrostatic interaction with ionic inducers[4-6,10] or by dispersing hydrophobic components in the bulk bilayer phase and resultant phase separation[7-9] are responsible for the liposome fusion. In earlier studies, meanwhile, homogeneous fusion of liposomes has been characterized by the growth of small vesicles to larger multicompartment liposomes, which is monitored indirectly by physical changes of membrane properties using various techniques such as NMR,[7,9] DSC,[5,6,8] turbidity,[12,13] luminescence assay,[14-16] or osmotic gradient measurements[4] and also visualized directly by electronmicrographic technique.[4,6,8,10]

In certain cases the liposome fusion also is accompanied by the leakage of contents in the interior core of vesicles.[4,9] It is, therefore, possible that appropriately prepared liposomes may be used to penetrate drugs into specific cell membranes through the liposome-cell membrane fusion. However, the details of the membrane fusion process are not yet well established. If it were clarified, therefore, one may conveniently control both the release of the liposome-encapsulated drugs and the delivery of drugs to the target organ.

In a recent paper we indicated that a functional surfactant bearing an imidazole moiety at the head group plays a role as a simple lipase model to cause chemical lysis of lecithin liposomes.[17] The chemical lysis could be monitored by following a leakage of amphiphilic marker dye, Bromothymol Blue (BTB), from the bulk bilayer phase to the exterior aqueous media.[17] Not only by the lysis of liposomal membranes but also even by physico-chemically perturbing the bilayer structure, BTB being entrapped closely to the membrane surface is released from the bilayer to the exterior. In this work we would like to describe the liposome-liposome fusion induced by several lipophilic

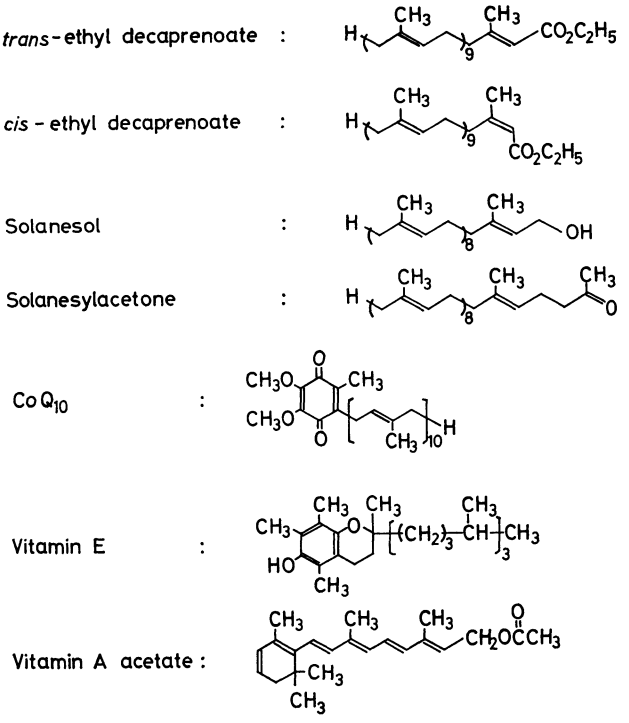

Fig. 1. Chemical structures of lipophilic agents employed in this work.

agents, where we adopted again the same marker-release technique to observe the fusion caused by the fluctuation of bilayer structure. A set of two different single compartment liposomes, the *A*-liposome encapsulating BTB and the *B*-liposome containing the neutral and lipophilic agents, respectively, was prepared. The liposome-liposome fusion can also be visualized directly by electron micrography. As the hydrophobic and neutral agents which are expected to accelerate the fusion, several kinds of polyisoprenoid pharmacological agents, *cis*- and *trans*-ethyl decaprenoate, solanesol, solanesylacetone, coenzyme Q_{10} (CoQ_{10}), vitamin E, and vitamin A acetate were employed (Fig. 1).

Experimental

Materials. All the lipophilic agents employed here were a gift from Eisai Co. Ltd., Tokyo. Egg yolk phosphatidylcholine (EL) was isolated and purified from egg yolk as described in Refs. 18 and 19. Purity of the lecithin was ascertained by TLC on silica-gel plates (Tokyo Kasei, S-160).[18,19] Bromothymol Blue was the same as that used in previous work.[17]

Preparation of Liposomes. Single and multicompartment liposomes containing BTB (*A*-liposome) or a lipophilic agent (*B*-liposome) were prepared independently according to the essentially same procedures as described in a previous paper.[17] A mixed thin film of egg phosphatidylcholine with or without cholesterol, the marker, or lipophilic agents was dispersed and swollen in 0.1 M aqueous citrate buffer solution (pH 5.0) containing 0.1 M NaCl to form multicompartment liposome, and then sonicated under nitrogen atmosphere to obtain single compartment liposomes. Single compartment liposomes were isolated by passing through a Sepharose 4B column[20] using 0.1 M NaCl as an eluant. Optically transparent liposome solutions were obtained by ultrafiltration using a Sartrius Membrane Filter SM 16315 equipped with a filter SM 11310 (0.05 μ), if necessary. By this technique, BTB was incorpotated in the bulk bilayers, not in the interior aqueous core.[17] Liposome-encapsulated BTB was determined spectrophotometrically ($\varepsilon_{617}=7.3\times 10$ $M^{-1}\cdot cm^{-1}$ at pH 7) after the gel-filtration, when liposomes were completely destructed by hydrolysis in strongly alkaline media at elevated temperature.[17] All the lipophilic agents employed are insoluble in water. They also were, therefore, almost quantitatively intercalated in the bulk phase of liposomal bilayers. Liposome concentrations were determined as inorganic phosphates by the modified Allen's method.[21] To obtain reliable data, for a series of experiments to compare each other, we prepared a large amount of concentrated stock solutions of liposomes as possible and diluted or devided them into several portion.

Typical Procedures to Monitor Bromothymol Blue Release upon Fusion. A 0.5 ml solution of the *A*-liposome, 1.5 ml of ethanol,[22] and 1.0 ml of aqueous buffered solution (phosphate, pH 7.0[23]) were placed in a thermoregulated cuvette cell and preincubated for 10 min at 25.0 °C unless otherwise stated. The BTB release induced by the addition of an appropriate amount of *B*-liposome solution was followed by monitoring the increase of intensity at 617 nm on a Hitachi 124 recording spectrophotometer.

During the preincubation, we could not find any spontaneous release of the marker. The percentage of BTB release was obtained by Eq. 1; where A is the absorbance at 617 nm, the subscripts t and 0 refer to the times of samples and to the instant when the *B*-liposome solution was added to the *A*-liposome solution, respectively. A_∞ stands for the absorbance of total amount of the liposome-encapsulated marker in *A*-liposome.

$$\%\ \text{BTB-release} = \frac{A_t - A_0}{A_\infty - A_0} \times 100 \qquad (1)$$

Maximum % release of BTB was the value over the plateau region after leveled off, usually after 60 min. All the runs were duplicated.

Results and Discussion

Fusion process of membranes, where the membrane of small vesicles is incorporated into larger membrane arrays, is one of the important problems to understand biological membranes.

Either when only *A*-liposome solution was incubated by itself or when a blank *B*-liposome containing no lipophilic agents was added to the preincubated *A*-liposome, no BTB release was observed at least in periods of several hours. This means that no fusion occurs. On the other hand, when an appropriate amount of liposome-encapsulated ethyl decaprenoate (a mixture of 15% *cis* and 85% *trans* isomers) was added into the *A*-liposome solution, enhanced BTB release upon the liposome-liposome fusion was observed. If we accept the mechanism of the liposomal fusion proposed by Papahadjopoulos and his co-workers,[5] it is reasonable to observe the marker release upon fusion. The liposome-liposome fusion accompanied by the BTB release was directly visualized by electron micrography,

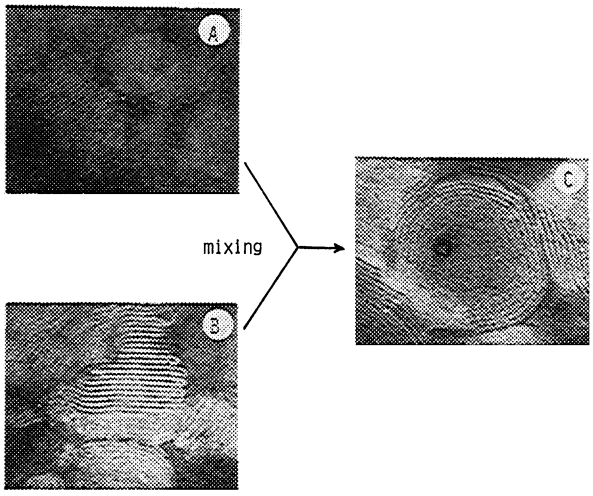

Fig. 2. Electron micrographic observation of the liposome-liposome fusion induced by a lipophilic agent, ethyl decaprenoate. Single compartment liposomes were prepared from egg phosphatidylcholine. A: The single compartment *A*-liposome containing BTB (0.82 mol %) and cholesterol (41.4 mol %). B: The single compartment *B*-liposome containing ethyl decaprenoate (28.2 mol%) and cholesterol (30.3 mol%). C: Multicompartment liposomes obtained by mixing *A*- and *B*-liposome solutions at 25 °C. All the liposome solutions were negatively stained with 2% (by wt.) aqueous sodium tungstophosphate solution. Measurements were run on a JEOL JEM 100 V electron microscope. Magnification ×250000.

which showed the evident growth of small single compartment liposomes to larger multilamellar liposomes (Fig. 2). The extent and the rate of fusion was comparable with those of the BTB release from the *A*-liposomes. The unstable state of membranes susceptible to fusion should makes the liposomal membranes highly permeable.[5] Several studies have shown that small, physiologically active, and lipophilic agents fluidize membranes.[24,25] Contrary to this, a few studies showed that lipophilic molecules may also exert a condensing effect on the liposomal bilayers.[26,27] The condensation in the bilayers may well create structural defects with resulting in fusion.[5,10] Increasing the content of the intercalated marker in the liposomal bilayer also will give rise to the instability of the membranes.[24,25] Therefore, the content of BTB in the *A*-liposomes was usually depressed less than 10 mol %. In any event, factors affecting the initial rate and the extent of the induced fusion by lipophilic agents are very complicated.

For investigating the lipophilic agent-induced fusion, all the following parameters must be perfectly controlled and kept satisfactorily constant; (i) the content of the marker in the *A*-liposome, (ii) the amount of lipophilic agents intercalated in the *B*-liposome, (iii) actual concentrations of *A*- and *B*-liposome, that is, the concentration ratio of *A*-liposome to *B*-liposome, and (iv) amount of multicompartment liposomes contaminated. Unfortunately, it was very difficult to establish

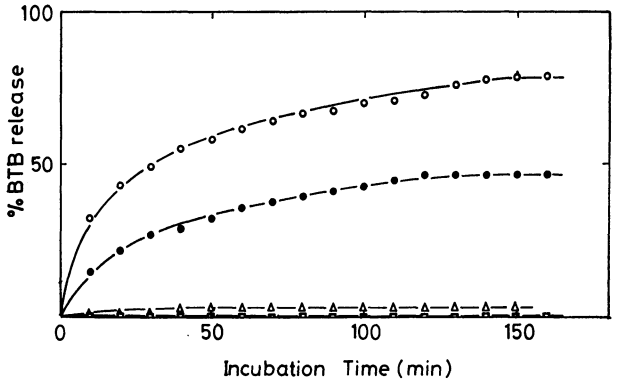

Fig. 3. Effect of cholesterol on the ethyl decaprenoate-induced fusion of egg lecithin single compartment liposomes at 25 °C and pH 7.0 (phosphate buffer). ○: *A*-Liposome consisting of 13.8 mM egg lecithin, 10.2 mM cholesterol, and 4.82 mM BTB was stimulated with *B*-liposome consisting of 1.15 mM egg lecithin, 0.83 mM cholesterol, and 0.78 mM ethyl decaprenoate. ●: *A*-Liposome consisting of 13.8 mM egg lecithin, 10.2 mM cholesterol, and 4.82 mM BTB was stimulated with *B*-liposome consisting of 1.15 mM egg lecithin and 0.78 mM ethyl decaprenoate *without* cholesterol. △: *A*-Liposome consisting of 13.8 mM egg lecithin and 4.82 mM BTB *without* cholesterol was incubated with *B*-liposome consisting of 1.15 mM egg lecithin, 0.83 mM cholesterol, and 0.78 mM ethyl decaprenoate. □: *A*-Liposome consisting of 13.8 mM egg lecithin, 10.2 mM cholesterol, and 4.82 mM BTB was incubated with a blank liposome consisting of 15.0 mM egg lecithin and 10.9 mM cholesterol without any lipophilic agent.

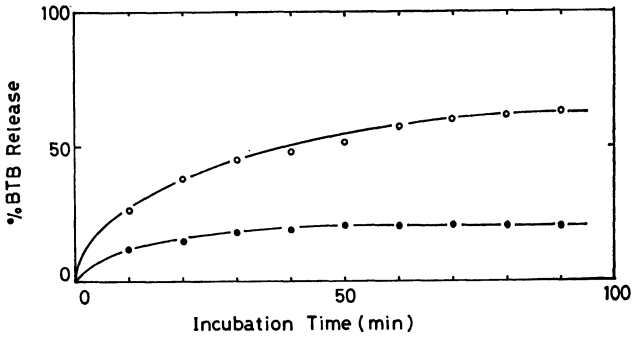

Fig. 4. Difference in efficiency of *cis*- and *trans*-ethyl decaprenoate on the liposome-liposome fusion as induced by lipophilic agents. *A*-Liposome consisting of 9.5 μM egg lecithin, 7.0 μM cholesterol, and 1.52 μM BTB was stimulated with *B*-liposomes consisting of 5.08 μM egg lecithin, 3.71 μM cholesterol, and 3.45 μM ethyl decaprenoate (*trans* (○) and *cis* (●) isomers) at 25.0 °C and pH 7.0.

the completely controlled conditions to satisfy all the above requirements. Therefore, we could discuss only the results obtained from a series of experiments employing the same liposome stock solution.

After the relatively rapid release of the marker at the initial stage, the marker release leveled off (Figs. 3 and 4). Since an excess surface free energy is the main thermodynamic driving force for fusion, the initial growth from small vesicles to diameters of negligible curvature results in the reduced surface free energy and the fusion will cease.[28,29] It is evident from the fact that we could not observe any fusion and BTB release when multicompartment liposomes were used. The rate-determining step of the fusion process is to be seen in the reaction which leads from the short-lived encounter complex of two colliding vesicles to the finally fused vesicle. According to Lawaczeck's theory[30] and if it were the liposome-liposome direct fusion in a spontaneous manner, therefore, one can establish a linear relationship between the initial rate of fusion and the square of the lecithin or liposome concentration. That is, under the assumption of a critical bilayer concentration,[30] the direct liposome fusion is considered as a reaction of second order in the lecithin or liposome concentration. If fusion is considered as one liposome is absorbed by another one,[31] however, the state of affairs may be different. In our present case, in addition, fusion is not spontaneous but induced by the lipophilic agent-encapsulated liposome and experiments were always carried out under conditions where the concentration of *A*-liposomes was higher than that of *B*-liposomes. Hence, we could not attain any linear relationship between the BTB release at a given interval and the product of concentrations of *A*- and *B*-liposomes.

At a given concentration of *A*-liposomes the maximum of % BTB release was directly affected by the concentration of *B*-liposomes (Table 1). Removing cholesterol from either side of liposomes significantly depress the fusion (Fig. 3). Its effect was more prominent on the *A*-liposome side, in which removal of cholesterol gave rise to the complete depression of the induced fusion. One role of cholesterol in membranes is to regulate the

degree of order and mobility of the acyl chains of lecithins as a modulator of the packing.[32-34] But, the orientation of the headgroup is not changed by the addition of cholesterol.[35] Above the phase transition temperature of lipids, addition of cholesterol to liposomes induces a higher molecular order in the bilayers[36] and reduces the mobility of the acyl chain.[32] In the bilayer phase, however, lipids and cholesterol are considered as totally and uniformly miscible.[37] It is certain, in any event, that the incorporation of cholesterol is considered as an important factor for fusion to initiate. Above phase transition temperature, changing the incubation temperature from 15 °C to 26 °C did not necessarily affect the extent of fusion. The concentration of *B*-liposomes rather than the incubation temperature largely affect the induced fusion.

Figure 4 indicates the difference in the efficiency on the induced fusion between *cis* and *trans* isomers of ethyl decaprenoate which were carefully isolated from the mixture by column chromatography. Very recently, Seelig and Seelig indicated that the incorporation of a *cis* double bond into a bilayer membrane leads to a general decrease in the hydrocarbon chain ordering in all parts of the bilayer and it seems to be accompanied by some increase in the rate of motion.[38] Another group found that most phospholipids derived from *cis*-parinaric acid shows the melting transition at about 1 °C lower than those from the *trans*-isomer.[39] *trans*-Parinaric acid preferentially associates with solid-phase lipid, while the *cis*-isomer shows a more equal distribution between solid and fluid lipids.[39] Judging from these previous findings,[39,40] the encapsulation of *cis*-ethyl decaprenoate is expected as more effective as to induce fusion. Contrary to what would be expected, however, *trans*-ethyl decaprenoate was more effective about three-fold than the *cis*-isomer. Though unfortunately we can not explain the reason at the present time, an interesting finding is that the antihypertensive activity of the *trans*-isomer, which was investigated *in vivo* using hypertensive rats, is higher than the *cis*-isomer.[40]

Table 1 reveals the BTB release induced by different lipophilic agent encapsulated liposomes. Spisni *et al.* recently investigated the interaction between ubiquinones and egg lecithin liposomes.[41] They found from ESR studies that the presence of ubiquinones induces the fluidity of membranes, as shown by the decrease of the $2T_{\parallel}$ parameter and the shorter correlation time of the spin-probe.[41] This means the disordering effect of the side chain of ubiquinones intercalating in the hydrocarbon region of the liposomal bilayer. As expected from the previous investigation,[41] CoQ_{10}, vitamin E, and vitamin A acetate significantly induced the liposome fusion (Table 1). Their effects were relatively large comparing with those of simple polyisoprenoids. Increasing the drug content in the *B*-liposome generally gave rise to the decrease of the apparent BTB release. When the content ratio of CoQ_{10} to egg lecithin in the *B*-liposome exceeded about 10 mol% (more than about 40 mol% for vitamines), some aggregates just like oil drops along with regular liposomes were visualized on their electron micrographies. This means that introducing a large amount of these agents causes the destruction of liposome structure itself. Relative efficiency of the BTB release upon the fusion as induced by lipophilic drugs was as follows: *cis*-ethyl decaprenoate<*trans*-ethyl decaprenoate<solanesyl-

Table 1. Maximum bromothymol blue (BTB) release upon fusion induced by lipophilic agent encapsulated liposomes under various conditions at pH 7.0

A-liposome[a]			*B*-liposome[a]			Temp °C	Maximum[b] BTB release %
[Lecithin] μM	[Cholesterol] μM	[BTB] μM	[Lecithin] μM	[Cholesterol] μM	[Additive] μM		
Ethyl decaprenoate[c]							
173 (1.0)	127 (0.73)	2.45 (0.014)	17.3 (1.0)	12.7 (0.73)	11.8 (0.68)	25.0	29
173 (1.0)	127 (0.73)	2.45 (0.014)	17.3 (1.0)	12.7 (0.73)	11.8 (0.68)	20.0	28
173 (1.0)	127 (0.73)	2.45 (0.014)	17.3 (1.0)	12.7 (0.73)	11.8 (0.68)	15.0	26
173 (1.0)	127 (0.73)	2.45 (0.014)	3.44 (1.0)	2.51 (0.73)	2.34 (0.68)	25.0	19
Solanesylacetone							
173 (1.0)	127 (0.73)	2.67 (0.015)	3.44 (1.0)	2.43 (0.71)	2.34 (0.68)	25.0	38
Solanesol							
173 (1.0)	127 (0.73)	2.45 (0.014)	3.44 (1.0)	2.57 (0.71)	2.34 (0.68)	25.0	45
22.7 (1.0)	7.57 (0.33)	17.1 (0.75)	13.6 (1.0)	4.53 (0.33)	1.49 (0.11)	25.0	33
CoQ_{10}							
22.7 (1.0)	7.57 (0.33)	17.1 (0.75)	13.6 (1.0)	4.53 (0.33)	1.49 (0.11)	25.0	45
Vitamin E							
22.7 (1.0)	7.57 (0.33)	17.1 (0.75)	13.6 (1.0)	4.53 (0.33)	1.49 (0.11)	25.0	69
Vitamin A acetate							
22.7 (1.0)	7.57 (0.33)	17.1 (0.75)	13.6 (1.0)	4.53 (0.33)	1.49 (0.11)	25.0	71

a) Numbers in parentheses are the mole ratio of the marker, lipophilic agent, or cholesterol to egg lecithins isolated as single compartment liposomes. All the concentrations are those in cuvette cell. b) The maximum BTB release was obtained at 60 min later after injection of the *B*-liposomes (see text). The marker releases were calculated by Eq. 1. Details are described under Experimental. c) Unless stated otherwise, ethyl decaprenoate was a mixture of 15% *cis* and 85% *trans* isomers.

acetone $<$ solanesol $\ll$ CoQ_{10} $<$ vitamin E $\simeq$ vitamin A acetate. Difference of these agents in the induced liposome fusion is difficult to argue at the present time. But, one possibility is that the structure of head group of polyisoprenoids and their analogues seems to act delicately on the local property of membrane surface. Liposomal bilayers are capable of a high degree of structural discrimination, and lends support to the hypothesis that the lipid phase of cell membranes may be the site of action of these lipophilic agents.

Our thanks are due to Mr. Kazuo Egashira for the partial assistance in experiments and to Mr. Yoshio Hara for his skilled technical assistance on the electron micrographic studies. Authors also acknowledge helpful discussions of Professor Hiroshi Kaneto, Department of Pharmacology, Faculty of Pharmaceutical Sciences, Nagasaki University, and Dr. Toji Konishi, Eisai Co. Ltd., Tokyo.

References

1) G. Poste and A. C. Allison, *Biochim. Biophys. Acta*, **300**, 413 (1973).
2) G. Gregoriadis, *N. Engl. J. Med.*, **295**, 704 (1976).
3) C. Taupin and H. M. McConnell, "Mitochondria Biomembranes," North-Holland Publishing Co., Amsterdam (1972), p. 219.
4) C. Miller, P. Arvan, J. N. Telford, and E. Racker, *J. Membr. Biol.*, **30**, 271 (1976).
5) D. Papahadjopoulos, W. J. Vail, C. Newton, S. Nir, K. Jacobson, G. Poste, and R. Lago, *Biochim. Biophys. Acta*, **465**, 579 (1977).
6) D. Papahadjopoulos, *J. Colloid Interface Sci.*, **58**, 459 (1977).
7) H. L. Kantor and J. H. Prestegard, *Biochemistry*, **14**, 1790 (1975).
8) H. L. Kantor, S. Mabrey, J. H. Prestegard, and J. M. Sturtevant, *Biochim. Biophys. Acta*, **466**, 402 (1977).
9) H. L. Kantor and J. H. Prestegard, *Biochemistry*, **17**, 3592 (1978).
10) A. L. Lau and S. I. Chan, *Biochemistry*, **13**, 4942 (1974).
11) F. J. Martin and R. C. MacDonald, *Biochemistry*, **15**, 321 (1976).
12) O. Avramovic-Zikic and K. Colbow, *Biochim. Biophys. Acta*, **512**, 97 (1978).
13) N. O. Petersen and S. I. Chan, *Biochim. Biophys. Acta*, **509**, 111 (1978).
14) R. W. Holz and C. A. Stratford, *J. Membrane Biol.*, **46**, 331 (1979).
15) D. Hoekstra, A. Yaron, A. Carmel, and G. Scherphof, *FEBS Lett.*, **106**, 176 (1979).
16) T. D. Ingolia and D. E. Koshland, Jr., *J. Biol. Chem.*, **253**, 3821 (1977).
17) J. Sunamoto, H. Kondo, and A. Yoshimatsu, *Biochim. Biophys. Acta*, **510**, 52 (1978).
18) A. J. De Konig, *J. Chem. Educ.*, **51**, 48 (1974).
19) B. J. White, C. L. Tripton, and M. Dressel, *J. Chem. Educ.*, **51**, 533 (1974).
20) J. Brunner, P. Skrabal, and H. Hauser, *Biochim. Biophys. Acta*, **455**, 322 (1976).
21) R. J. L. Allen, *Biochem. J.*, **34**, 858 (1940).
22) To obtain a completely transparent solution for the optical purpose, ethanol was introduced. Under conditions adopted, that the addition of ethanol does not cause any enhanced fusion of liposomes was of course ascertained beforehand.
23) At this stage, a transmembrane pH-gradient (interior pH 5.0 and exterior pH 7.0) was retained.[17]
24) J. R. Trudell, W. L. Hubbell, and E. N. Cohen, *Biochim. Biophys. Acta*, **291**, 321 (1973).
25) D. K. Lawrence and E. N. Gill, *Mol. Pharmacol.*, **11**, 280 (1975).
26) D. K. Lawrence and E. N. Gill, *Mol. Pharmacol.*, **11**, 595 (1975).
27) M. J. Neal, K. W. Butler, C. F. Polnaszek, and I. C. P. Smith, *Mol. Pharmacol.*, **12**, 144 (1976).
28) C. Miller and E. Racker, *J. Membrane Biol.*, **26**, 319 (1976).
29) N. P. N. Gent and J. H. Prestegard, *Biochemistry*, **13**, 4027 (1974).
30) R. Lawaczeck, *J. Colloid Interface Sci.*, **66**, 247 (1978).
31) G. Poste and A. C. Allison, *Biochim. Biophys. Acta*, **300**, 421 (1973).
32) K. -Y. Y. Pang and K. W. Miller, *Biochim. Biophys. Acta*, **511**, 1 (1978).
33) S. Kawato, K. Kinoshita, Jr., and A. Ikegami, *Biochemistry*, **17**, 5026 (1978).
34) S. J. Shattil and R. A. Cooper, *Biochemistry*, **15**, 4832 (1976).
35) T. J. McIntosh, *Biochim. Biophys. Acta*, **513**, 43 (1978).
36) Y. Shimoyama, L. E. G. Eriksson, and A. Ehrenberg, *Biochim. Biophys. Acta*, **508**, 213 (1978).
37) T. Y. Tsong, *Biochemistry*, **14**, 5415 (1975).
38) A. Seelig and J. Seelig, *Biochemistry*, **16**, 45 (1977).
39) L. A. Sklar, B. S. Hudson, and R. D. Simoni, *Biochemistry*, **16**, 819 (1977).
40) Personal communication from Dr. Toji Konishi, Eisai Co. Ltd.
41) A. Spisni, L. Masotti, G. Lenaz, E. Bertoli, G. F. Pedulli, and C. Zannoni, *Arch. Biochem. Biophys.*, **190**, 454 (1978).