[Chem. Pharm. Bull. 36(10)4192—4198(1988)]

Alteration of Immunolysis Reaction on Liposome Membrane by Various Cholesterol Analogues

USA GLAGASIGIJ, YUKIO SATO,* and YASUO SUZUKI

Pharmaceutical Institute, Tohoku University, Aobayama, Sendai 980, Japan

(Received March 22, 1988)

Complement-mediated immunolysis was employed to examine the effect of incorporation of various cholesterol analogues, having a terminal hydroxyl group or terminal primary amine group at the 3-position of the cholestene nucleus, into the bilayer membrane of haptenated reverse-phase evaporation vesicles. An enhancement of immunolysis was observed when triethoxycholesterol (analogue II) was incorporated, while no measurable change was detected when the chain length of the substituted groups in hydroxy cholesterols was shorter than that of analogue II. However, for amino cholesterol analogues, a remarkable decrease in immunolysis was seen. These results may arise from changes of membrane properties such as bilayer fluidity, lateral hapten mobility and complement fixation.

Keywords——immunoliposome; immunolysis; calcein; theophylline; cholesterol analogue

Introduction

Liposomes have been used as a model of biological membranes in the fields of biology and medicine.[1,2] In immunoassay, a minute amount of substances such as drugs,[3,4] antigens,[5] or antibodies[6,7] in biological fluids can be detected by immunolysis of haptenated vesicles.

In a previous report,[8] we described a highly sensitive assay of theophylline by using complement-mediated immunolysis of liposomes entrapping with a fluorescent probe, calcein. Although a nanomolar range of theophylline could be detected by this method, the system had the drawback of limited release of trapped marker, as is usually observed in the immunolysis of large liposomes.[2,8,9] However, the physical properties of liposomal membranes can be altered by an appropriate modification of vesicle components.[10,11] Cholesterol analogues were successfully used by Patel *et al.*[12,13] for enhancement of liposome stability *in vitro* and *in vivo*. These kinds of analogues differ from cholesterol in the 3β-OH head group (Fig. 1) resulting in alterations of charge and polarity. Thus, we examined the influence of incorporation of cholesterol analogues into the haptenated liposomal membrane on the immunolysis, with the aim of improvement of the sensitivity and reproducibility of the assay. The present paper shows that the incorporation of triethoxycholesterol results in a strong immunolysis reaction.

Experimental

Materials——Dilauroylphosphatidylcholine (DLPC) was obtained from Nippon Oil & Fats Co., Ltd., Tokyo, cholesterol from Koso Chemical Co., Ltd., Tokyo, and *l*-α-phosphatidylethanolamine and calcein from Sigma Chemical Co., St. Louis. Guinea pig complement was from the Research Laboratory of Microbiology, Osaka University. All other reagents used were of analytical grade.

Preparation of Antibody——Antiserum to theophylline was raised in rabbit by immunization with a conjugate of 8-(3-carboxypropyl)-1,3-dimethylxanthine and bovin serum albumin (BSA).[14]

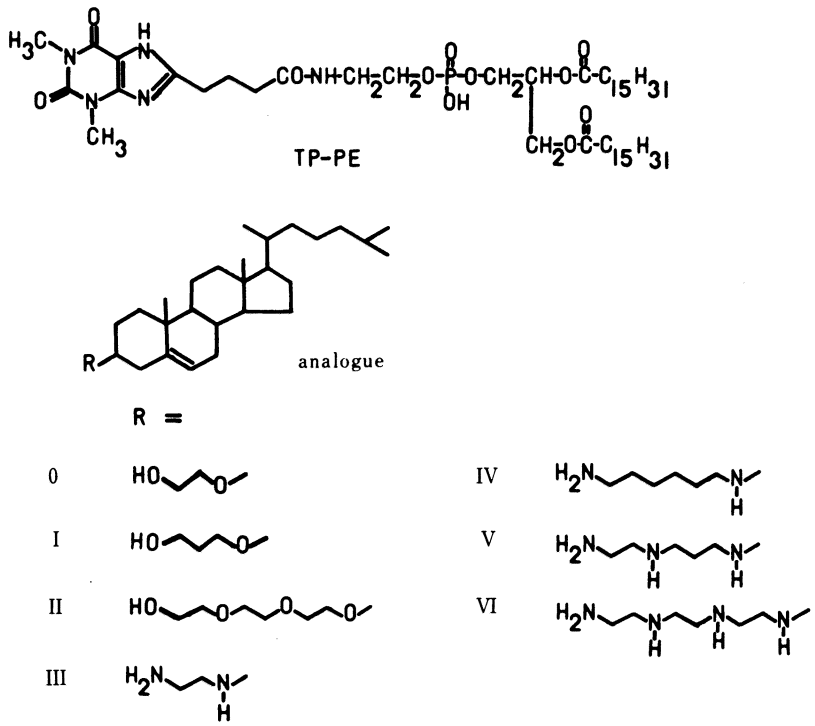

Fig. 1. Chemical Structures of TP–PE Hapten and Various Synthetic Cholesterol Analogues (Analogue 0—Analogue VI)

Synthesis of Hapten and Cholesterol Analogues——8-(3-Carboxypropyl)-1,3-dimethylxanthine–phosphatidylethanolamine conjugate (TP–PE) was synthesized as described in the previous paper.[8] Cholesterol analogues were synthesized according to the method of Patel *et al.*[12,13] All cholesterol analogues were further purified by chromatography on a column of silica gel, and eluted with a gradient mixture of chloroform and methanol. The chemical structures and abbreviations of TP–PE and cholesterol analogues are shown in Fig. 1. The abbreviations of the analogues reflect the chain length of the substituent and the nomenclature of Patel *et al.*[13]

Preparation of Haptenated Liposomes——Reverse-phase evaporation vesicles (REVs) composed of phosphatidylcholine, cholesterol and TP–PE hapten at a molar ratio of 1 : 1 : 0.01 (control vesicles) were prepared in the presence of 58 mM calcein according to the methods described by Szoka and Papahadjopoulos[15] and Düzgünes *et al.*[16] Liposomes composed of various cholesterol analogues were prepared by further adding individual cholesterol analogues at molar ratios from 0.25 to 2.0 relative to DLPC.

Measurements of Encapsulation Efficiency and Capture Volume——Encapsulation efficiency was determined by the method of Szoka and Papahadjopoulos[15] and capture volume of REVs was measured by the method of Düzgünes *et al.*[16]

Standard Assay System——Immunolysis of liposomes was determined by measuring the fluorescence due to released calcein as previously described in detail.[8] In brief, 20 μl of 0.46 mM liposomal phospholipid suspension was incubated with 125 μl of antibody solution and 80 μl of complement solution (both concentrations set at the plateau of antibody-dependent lysis) in a final volume of 1 ml in 20 mM Tris–HCl, 150 mM NaCl buffer (pH 7.4) containing 0.5 mM $MgCl_2$ and 0.15 mM $CaCl_2$ ($Tris^{2+}$ buffer). After further incubation with shaking at 25°C for 30 min, the reaction mixtures were diluted with 1.5 ml of $Tris^{2+}$ buffer and the fluorescence intensity was determined by excitation at 494 nm, with emission at 512 nm. The total calcein fluorescence in liposomes was determined after the addition of sodium deoxycholate to a final concentration of 0.19%.

The homogeneity and efficiency of incorporation of cholesterol and cholesterol analogues into the lipid mixture were tested by using another type of liposome. Sonicated liposomes (SUVs) of the same composition as REVs were prepared and separated from interfering substances by passing them through a Sepharose CL-4B column. High lipid recovery (more than 90%) was observed from both REVs and SUVs. Incorporation of cholesterol and cholesterol analogues was checked by measuring electron spin resonance (ESR) spectra. That is to say, the spectra of the REVs and SUVs showed changes depending upon the amounts of cholesterol and analogues (data not shown).

In kinetic experiments on liposome immunolysis,[11,17–19] the initial change of fluorescence was recorded at 25°C

immediately after the addition of complement, without further incubation. The increase of fluorescence per min was measured. The value of the kinetic parameter (% release/min) was obtained on the basis of total fluorescence observed after addition of sodium deoxycholate.

Spectral Measurements——The fluorescence spectra were taken on a JASCO FP-550 recording fluorometer at a controlled temperature.

Results

Effect of Cholesterol Analogues on Solute Entrapment of Liposomes

Incorporation of cholesterol analogues into the liposomal membrane resulted in various changes of parameters depending on the molecular species and amounts. Figures 2a and b show the effect of cholesterol analogues on encapsulation efficiency and capture volume. By incorporating hydroxy cholesterol analogues into haptenated vesicles at low molar ratios, the encapsulation efficiency and capture volume were significantly increased. In contrast, vesicles incorporating amino cholesterol analogues showed marked decreases in both parameters. However, the incorporation thresholds of hydroxy cholesterol analogues into the liposomal membrane were found at the molar ratio of 1.5 for analogues 0 and I, and at the molar ratio of 2.0 for analogue II. A further increase of the molar ratio of hydroxy cholesterol analogues caused the production of a highly viscous gel in the process of liposome formation. In the case

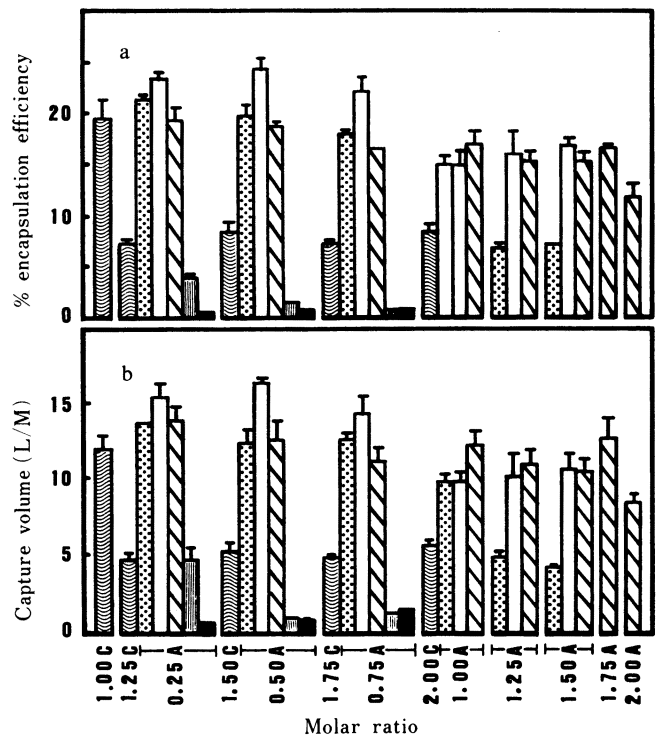

Fig. 2. Dependence of REVs Solute Entrapment on Concentration of Cholesterol and Cholesterol Analogues

REVs were prepared from a mixture of DLPC–cholesterol (1 : 1—2 molar ratio), C; or DLPC–cholesterol–analogue (1 : 1 : 0.25—2.00 molar ratio), A.

(a) Encapsulation efficiency: the percentage of the initial aqueous phase that becomes entrapped in liposomes.

(b) Capture volume, L/M, in liters of aqueous space per mol of phospholipid.

▨, cholesterol; ⊡, analogue 0; □, analogue I; ▧, analogue II; ▩, analogue III; ■, analogue VI.

Each point represents the mean of at least three samples ± S.D.

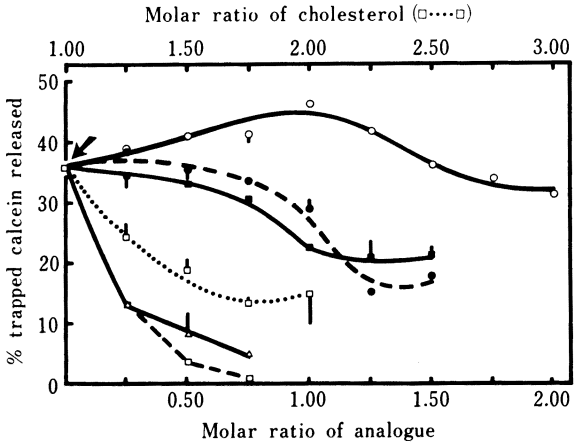

Fig. 3. Effect of Concentration of Cholesterol and Cholesterol Analogues on Immunolysis of Haptenated Liposomes

REVs were prepared from mixture of DLPC–cholesterol (1 : 1—2 molar ratio) or DLPC–cholesterol–analogue (1 : 1 : 0.25—2.00 molar ratio). The assay mixture was composed of 20 μl of liposomal suspension, 125 μl of antibody solution and 80 μl of complement solution. The arrow indicates control vesicles (DLPC–cholesterol = 1 : 1).

□···□, cholesterol; ●---●, analogue 0; ■—■, analogue I; ○—○, analogue II; □---□, analogue III; △—△, analogue VI.

Each point represents the mean of at least three samples ± S.D.

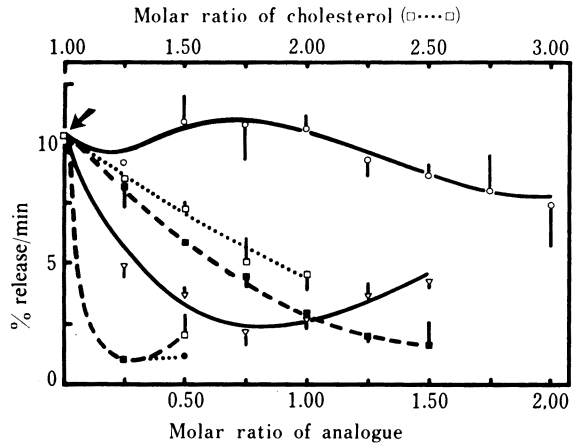

Fig. 4. Effect of Concentration of Cholesterol and Cholesterol Analogues on Immunolysis Rate of Haptenated Liposomes

REVs composed of DLPC–cholesterol (1 : 1—2 molar ratio) or DLPC–cholesterol–analogue (1 : 1 : 0.25—2.00 molar ratio). The arrow indicates control vesicles (DLPC–cholesterol = 1 : 1).

□···□, cholesterol; ▽—▽, analogue 0; ■---■, analogue I; ○—○, analogue II; ●···●, analogue III; □---□, analogue VI.

Each point represents the mean of at least three samples ± S.D.

of amino cholesterol analogues, REVs could be prepared from analogues III and VI only, and up to molar ratio of 0.75, with low encapsulation efficiency. Further addition of analogue III or VI resulted in aggregation. From the lipid mixtures composed of DLPC, cholesterol and analogues IV and V, liposomes could not be formed at all. When the amount of cholesterol itself was increased, a substantial reduction in both encapsulation efficiency and capture volume was observed, and reached the threshold at a molar ratio of 1 : 2 (DLPC : cholesterol).

Effect of Incorporation of Analogue II on Antibody-Dependent Immunolysis of Haptenated Vesicles

An increase of the amount of analogue II in the liposomal membrane affected the immunolytic release of calcein from liposomes (Fig. 3). The calcein release in the absence of any cholesterol analogue (control vesicles), 36% ($p < 0.05$), was increased to 46% ($p < 0.05$) by the addition of 1.0 molar ratio of analogue II. A further increase of analogue II resulted in a gradual decrease of immunolysis. No noticeable change in marker release was observed up to molar ratio of 0.5 for analogues 0 and I, and further addition resulted in a decline of the immunolysis. A remarkable reduction in immunolysis was observed by using amino cholesterol analogues. An increase of the amount of cholesterol itself also resulted in a reduction of immunolysis. No detectable release was observed upon substitution of normal rabbit serum for antibody, demonstrating the high specificity and stability of haptenated

vesicles.

Kinetics of liposome immunolysis in the presence of a variety of cholesterol analogues are shown in Fig. 4, as reported elsewhere.[11,17-19] The relative rate of immunolysis (% release/min) was in the order of II > cholesterol > I > 0 > III—VI at every molar ratio except for a slightly rise with analogue 0 at molar ratios above 1.0. Increased incorporation of analogue generally resulted in a reduction of the rate of immunolysis except for analogue II, for which the rate remained nearly unchanged up to 1.00 molar addition and decreased slowly above that amount. A decline of immunolysis rate was also observed on increasing the amount of cholesterol itself from 1 : 1 (DLPC : cholesterol) to 1 : 2.

Discussion

Several investigators have suggested that the interaction of membranous hapten with the antibody might be affected by membrane composition, which influences the projection and mobility of the hapten.[11,20] Haptens on a liposomal membrane composed of 'fluid' phospholipids (low transition temperature) effectively reacted with antibody and complement due to their greater lateral mobility, which contributes to the attachment of the antibody to form complexes.[18,21,22] Cholesterol is one of the major components in biological membranes, where its role is to regulate the organization and fluidity of lipids, and to control the interactions between membranous constituents and extracellular components.[21,23] In our previous study,[8] stable haptenated vesicles were obtained from a lipid mixture composed of equimolar amounts of cholesterol and DLPC, in agreement with other investigators.[7,24] Although the vesicles composed of DLPC and cholesterol in 1 : 0.5 and 1 : 0.75 molar ratios showed high sensitivity in immunolysis, $40.4 \pm 3.3\%$ and $34.4 \pm 2.0\%$, respectivey, the vesicles were unstable under the assay conditions. Further, significant leakage of calcein was observed in the control experiment in the presence of complement alone, *e.g.*, $37.8 \pm 2.0\%$ and $22.2 \pm 0.8\%$ from vesicles incorporating cholesterol at 0.5 and 0.75 molar ratios to DLPC, respectively. A large amount of untrapped marker ($17.5 \pm 0.1\%$ in Tris buffer) was also found in the vesicles composed of DLPC and cholesterol at 1 : 0.5 molar ratio. Thus, the liposomes prepared with cholesterol at less than 1.0 molar ratio could not be used in our assay system.

On the other hand, several workers have reported that liposomes could be prepared from mixtures of phosphatidylcholine and cholesterol up to a molar ratio of 1 : 2[25] or 1 : 3[26] in small unilamellar vesicles and up to 1 : 3 in large multilamellar vesicles.[27] In the present study, we also examined the effect of incorporating of high concentrations of cholesterol (DLPC : cholesterol = 1 : 1.25 to 1 : 2) in haptenated reverse-phase evaporation vesicles on the immunolysis. As shown in Fig. 3, a decrease in immunolysis was obtained. Nevertheless, the incorporation of analogue II gave a high immunolysis reaction at DLPC : cholesterol : analogue II = 1 : 1 : 1 molar ratio. This might be due to the perturbation of the surface of the lipid bilayer due to the steric effect of the bulky hydrophilic head group of analogue II, reducing the phospholipid's hydrocarbon chain contacts. This would give rise to fluidization of the membrane[28,29] so that the hapten might be sequestered at the membrane surface, exposed to antibody binding, and to reaction with complement.[22,30,31] However, at higher concentrations of analogue II (molar ratio to DLPC more than 1.0), the immunolysis was decreased due to tighter packing, which would cause greater membrane stiffness and reduction of the hapten lateral mobility.[22,30] For analogues 0 and I, whose chemical structures are very similar to that of cholesterol itself, the immunolysis was also decreased.

We also examined the electron spin resonance (ESR) spectra of REVs incorporating hydroxy cholesterol analogues by using the 3-nitroxide of cholestane as a spin probe.[32] Evidence of increasing membrane fluidity in the liposomal membrane incorporating analogue II was revealed around molar ratios of 0.75—1.25 from the ESR curve, in contrast with the

restricted mobility of DLPC hydrocarbon chains observed with analogues 0 and I (data not shown).

In the case of amino cholesterol analogues, liposomes could be formed only wtih analogues III and VI at molar ratios to cholesterol up to 0.75. No liposomes could be prepared in the presence of analogue IV or V. This might be due to the interaction between the amino group of cholesterol analogues and the carboxy group of calcein.[33] Interaction might also occur between the amino group and lipid phosphate group,[13] because a color change from white to purple and then red was observed in REVs without calcein.

A high association rate between hapten and antibody was observed in the haptenated vesicles incorporating analogue II at every molar ratio tested, as illustrated in Fig. 4. Since the rate and strength of the antigen–antibody binding depended on the lipid composition of the vesicles,[11,18] it was apparent that binding was favorable in that membrane.

Haptenated vesicles prepared from the mixture of DLPC and hydroxy cholesterol analogues (1 : 1 molar ratio) also reacted with antibody and complement and released calcein (40%, 39%, and 36% for analogues II, I, and 0, respectively). However, significant leakage of calcein was observed in the absence of antibody in REVs composed of DLPC and analogue II. Reducing the amount of cholesterol in vesicles to less than 1.0 (*e.g.*, DLPC : cholesterol : analogue II = 1 : 0.5 : 1 molar ratio) also resulted in significant leakage of marker (15.8% calcein release in the presence of complement alone), though high sensitivity in immunolysis was obtained (47.4%). The presence of cholesterol was necessary for the present studies.

Using REVs composed of DLPC, cholesterol and hydroxy cholesterol analogues, no significant leakage of calcein was observed in the assay in the absence of antibody, and the REVs could be stored under a nitrogen atmosphere more than one month.

The enhancement in immunolytic potency and reproducibility by the use of liposomes incorporating analogue II presented here should provide a unique material for application in the field of clinical immunoassay. Although complete release of marker still cannot be obtained, the higher amplification of immunolysis observed in this system should result in increased sensitivity of detection with high accuracy and precision. An assay of theophylline in the presence of analogue II will be reported in a following paper. Further variation in membrane composition may result in even more convenient membrane properties. The present results should be useful in various fields of membrane biology.

References

1) K. Yagi (ed.), "Medical Application of Liposomes," Japan Scientific Societies Press, Tokyo, 1986.
2) R. E. Silversmith and G. L. Nelsestuen, *Biochemistry*, **25**, 7717 (1986).
3) J. W. Freytag and W. J. Litchfield, *J. Immunol. Methods*, **70**, 133 (1984).
4) W. J. Litchfield, J. W. Freytag, and M. Adamich, *Clin. Chem.*, **30**, 1441 (1984).
5) M. Umeda, Y. Ishimori, K. Yoshikawa, M. Takada, and T. Yasuda, *J. Immunol. Methods*, **95**, 15 (1986).
6) D. I. Slovick, J. Saida, R. P. Lisak, and A. Schreiber, *J. Immunol. Methods*, **39**, 31 (1980).
7) Y. Ishimori, T. Yasuda, T. Tsumita, M. Notsuki, M. Koyama, and T. Tadakuma, *J. Immunol. Methods*, **75**, 351 (1984).
8) U. Glagasigij, Y. Sato, and Y. Suzuki, *Chem. Pharm. Bull.*, **36**, 1086 (1988).
9) R. L. Richards, R. C. Habbersett, I. Scher, A. S. Janoff, H. P. Schieren, L. D. Mayer, P. R. Cullis, and C. R. Alving, *Biochim. Biophys. Acta*, **855**, 223 (1986).
10) K. Agarwal, A. Bali, and C. M. Gupta, *Biochim. Biophys. Acta*, **856**, 36 (1986).
11) A. Petrossian and J. C. Owicki, *Biochim. Biophys. Acta*, **776**, 217 (1984).
12) K. R. Patel, M. P. Li, J. R. Schuh, and J. D. Baldeschwieler, *Biochim. Biophys. Acta*, **797**, 20 (1984).
13) K. R. Patel, M. P. Li, J. R. Schuh, and J. D. Baldeschwieler, *Biochim. Biophys. Acta*, **814**, 256 (1985).
14) H. Itagaki, Y. Hakoda, Y. Suzuki, and M. Haga, *Chem. Pharm. Bull.*, **31**, 1283 (1983).
15) F. Szoka and D. Papahadjopoulos, *Proc. Natl. Acad. Sci. U.S.A.*, **75**, 4194 (1978).
16) N. Düzgünes, J. Wilschut, K. Hong, R. Fraley, C. Perry, S. F. Daniel, T. L. James, and D. Papahadjopoulos,

Biochim. Biophys. Acta, **732**, 289 (1983).
17) M. Smolarsky, D. Teitelbaum, M. Sela, and C. Gitler, *J. Immunol. Methods*, **15**, 255 (1977).
18) R. Luedtke and F. Karush, *Biochemistry*, **21**, 5738 (1982).
19) R. J. Y. Ho, H. P. Ting-Beall, B. T. Rouse, and L. Huang, *Biochemistry*, **27**, 500 (1988).
20) K. Takeshita, H. Utsumi, and A. Hamada, *Biophys. J.*, **52**, 187 (1987).
21) P. Brûlet and H. M. McConnell, *Proc. Natl. Acad. Sci. U.S.A.*, **73**, 2977 (1976).
22) J. T. Lewis and H. M. McConnell, *Ann. N.Y. Acad. Sci.*, **308**, 139 (1978).
23) T. Suzuki, H. Utsumi, K. Inoue, and S. Nojima, *Biochim. Biophys. Acta*, **644**, 183 (1981).
24) C. Kirby, J. Clarke, and G. Gregoriadis, *Biochem. J.*, **186**, 591 (1980).
25) C. Horwitz, L. Krut, and L. Kaminsky, *Biochim. Biophys. Acta*, **239**, 329 (1971).
26) R. A. Cooper, M. H. Leslie, S. Fischkoff, M. Shinitzky, and S. J. Shattil, *Biochemistry*, **17**, 327 (1978).
27) E. Arakawa, Y. Imai, H. Kobayashi, K. Okumura, and H. Sezaki, *Chem. Pharm. Bull.*, **23**, 2218 (1975).
28) F. T. Presti and S. I. Chan, *Biochemistry*, **21**, 3821 (1980).
29) P. Brûlet and H. M. NcConnell, *Biochemistry*, **16**, 1209 (1977).
30) D. Marsh and I. C. P. Smith, *Biochim. Biophys. Acta*, **298**, 133 (1973).
31) G. M. K. Humphries and H. M. McConnell, *Proc. Natl. Acad. Sci. U.S.A.*, **74**, 3537 (1977).
32) U. Glagasigij, Y. Sato, and Y. Suzuki, *Chem. Pharm. Bull.*, **36**, 1589 (1988).
33) T. M. Allen, "Liposome Technology," Vol. III, ed. by G. Gregoriadis, CRC Press, Inc., Florida, pp. 177—182 (1984).