Adsorption of Pyranine onto Cationic Liposomal Membranes as Evidenced by Fluorescence Polarization†

Tadashi NOMURA, Hiroki KONDO, and Junzo SUNAMOTO*

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

(Received October 21, 1980)

Synopsis. A trianionic fluorescent probe, pyranine, was found to bind to positively charged liposomes as evidenced by a linear increase in the fluorescence polarization with the liposome concentration. Addition of perchlorate ion reduced the polarization of fluorescence, suggesting that perchlorate and pyranine compete for the same binding site on the surface of cationic liposomal membranes.

A knowledge of properties of membrane surface is essential to understand various phenomena occurring on membranes, because in biological systems most of events begins with the adsorption of a substance onto and permeation into membranes.[1] The surface of liposomal membranes as well as natural biomembranes has been characterized by a variety of techniques such as NMR[2] or neutron diffraction.[3] A focus has been on the negatively charged membranes to which cations bind through electrostatic interaction with a different degree of specificity.[4-6] In this work we would like to describe the interaction of positively charged liposomes with an anionic fluorescent probe, pyranine (trisodium 8-hydroxy-1,3,6-pyrenetrisulfonate), by means of fluorescence polarization. An advantage of utilizing this particular probe lies in the fact that it emits strong fluorescence at 510 nm and that it can bind to a charged membrane surface through the electrostatic interaction.[7,8] When the probe adsorbs onto the membrane surface the molecular motion of the probe will be depressed, possibly giving rise to a significant increase in the polarization of fluorescence.

Experimental

Pyranine was obtained from Eastman Kodak Co. and its purity was assessed before use by TLC on silica gel in butanol–water (6 : 1 by volume). Its concentration was determined on the basis of molar extinction coefficient of 1.02×10^4 M^{-1} cm^{-1} (1 M = 1 mol dm^{-3}) at 405 nm. Single-compartment liposomes of egg lecithin were prepared as described previously.[9,10] In the preparation of positively charged liposomes, hexadecylamine (20 mol% to lecithin) was added.[9] The pH of the liposome suspension was adjusted to 5 with either 0.6 mM CH_3CO_2H–1.4 mM CH_3CO_2Na or 5.4 mM $HClO_4$–9.6 mM $NaClO_4$. Fluorescence spectra were taken on a Hitachi 650-10 spectrofluorometer with 1 nm bandpaths for both excitation and emission sides. Fluorescence polarization was determined on a Union FS-501 fluorescence depolarization instrument which was connected to a Sord Microcomputer M 200 Mark II. Pyranine was excited at 405 nm and its fluorescence polarization was determined at 510 nm[11] The vertical and horizontal components of the emission were funneled through a Y-46 cut-off filter (50% transmittance at 460 nm). Fluorescence polarization is defined by Eq. 1:[9]

$$p = \frac{I_{VV} - C_f \cdot I_{VH}}{I_{VV} + C_f \cdot I_{VH}} \quad (1) \qquad C_f = I_{HV}/I_{HH},$$

where C_f is the grating correction factor[11] and I stands for the collected intensity of fluorescence at parallel and perpendicular positions of the polarizer. Subscripts V and cH refer to vertical and horizontal beams, respectively. For example, I_{VH} means the intensity of horizontal component of fluorescence when excited by the vertically polarized beam. All measurements were made at 25.0 °C under aerobic conditions.

Results and Discussion

The polarization of pyranine fluorescence was determined in the presence of neutral or positively charged liposomes in an aqueous buffered solution (pH 5) containing acetate or perchlorate ions (Fig. 1).

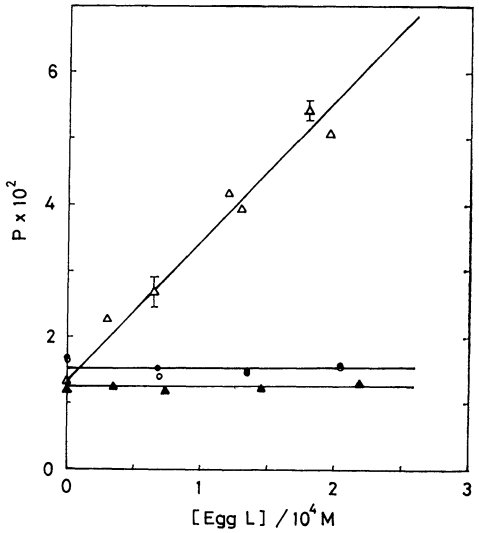

Fig. 1. Fluorescence polarization of pyranine as a function of egg lecithin concentration at pH 5 and 25.0 °C. Experiments were carried out using neutral liposomes in an aqueous acetate (▲) or perchlorate (●) solution and positively charged liposomes in an aqueous acetate (△) or perchlorate (○) solution. The concentration of pyranine was 2×10^{-6} M in all the runs.

For neutral liposomes the polarization did not vary over the liposome concentration range studied. This trend was common to the two salts adopted. These results indicate that no significant interaction takes place between pyranine and neutral liposomes. In the case of cationic liposomes in an acetate buffer, on the other hand, the polarization of pyranine fluorescence increased linearly with increasing liposome concentration. However, this was not the case for the perchlorate solution, suggesting that perchlorate ion interferes with the adsorption of pyranine onto the liposome surface. This was confirmed by the following experiment. When sodium perchlorate was further added to a cationic liposome suspension containing pyranine and acetate ion, the polarization decreased with an increase in the

† Liposomal Membranes, Part X from this laboratory.

Table 1. Effect of perchlorate anion on the fluorescence polarization of pyranine in the egg lecithin single-walled and positively charged liposome suspension at 25.0 °C and pH 5[a)]

$[NaClO_4]$/M	$p \times 10^2$
0	5.0(1.1)[b)]
2.0×10^{-3}	2.9
6.1×10^{-3}	1.9(1.2)

a) A liposome suspension contains 0.10 M NaCl, 1.4 mM CH_3CO_2Na, and 0.6 mM CH_3CO_2H. [Pyranine] $=2.0 \times 10^{-6}$ M; [Egg L] $=2.4 \times 10^{-4}$ M. b) Numbers in parentheses are p-values of pyranine in the absence of liposomes.

perchlorate concentration (Table 1).

From the result that the polarization of pyranine increases in the presence of cationic liposome, it is now evident that pyranine binds to the surface of positively charged liposomes through the electrostatic interaction and consequently the molecular motion of the probe is considerably hindered. It is of special interest that perchlorate ion competes for the same binding site whereas acetate ion does not. This difference seems to arise from the difference in the mode of hydration to these anions. It is presumed that the hydration to perchlorate is weaker than that to acetate, because the charge delocalization is more extensive for the former.[12)] This means that the weakly hydrated perchlorate ion may more easily form an ion pair with (more strongly binds to) the positive site of liposomal membranes. Certainly, the binding of these anions to positively charged liposomes follows a closely similar order to that for their interaction with anion exchange resins[13)] or acetyltetraglycine ethyl ester;[14)] perchlorate> sulfonate>acetate. The high lyophilicity as well as trianionic character of pyranine may make the probe to show the affinity for the cationic membrane surface.

It should be mentioned that the emission maxima and intensity of fluorescence from pyranine were not much affected upon the binding to liposomes. Thus the relative ratio of fluorescence intensity in the presence and absence of liposomes, I/I_0, changed by less than 10% over the egg lecithin concentration range $\approx$400 μM. The relative intensity was also independent of the probe concentration at least in a range of 1—5 μM. These results imply that quenching of pyranine fluorescence upon the binding to liposomes is negligible under the present experimental conditions. This is in contrast to the previous findings in which the concentration quenching of fluorescence was observed through the ionic probe aggregation on the surface of charged membranes.[15,16)] This also supports the contention that hexadecylamine molecules are dispersed evenly in the phospholipid bilayers and that no significant phase separation occurs even by the adsorption of the polyanionic probe.

We are grateful to Dr. Kaoru Tsujii, Tochigi Research Laboratories, Kao Soap Co. Ltd., for his helpful comments. This work was supported by a Grant-in-Aid for Scientific Research No. 447068, from the Ministry of Education Science and Culture.

References

1) P. L. Yeagle, *Acc. Chem. Res.*, **11**, 321 (1978).
2) J. Seeling, *Biochim. Biophys. Acta*, **515**, 105 (1978).
3) B. P. Schoenborn, *Biochim. Biophys. Acta*, **457**, 41 (1976).
4) R. J. Kurland, M. Hammoudah, S. Nir, and D. Papahadjopoulos, *Biochem. Biophys. Res. Commun.*, **88**, 927 (1979).
5) M. Eiserberg, T. Gresalfi, T. Riccio, and S. McLaughlin *Biochemistry*, **18**, 5213 (1979).
6) J. Sunamoto and M. Shironita, *Bull. Chem. Soc. Jpn.*, **53**, 2778 (1980).
7) K. Kano and J. H. Fendler, *Biochim. Biophys. Acta*, **509**, 289 (1978).
8) T. Nomura, J. R. Escabi-Perez, J. Sunamoto, and J. H. Fendler, *J. Am. Chem. Soc.*, **102**, 1484 (1980).
9) J. Sunamoto, K. Iwamoto, H. Kondo, and S. Shinkai, *J. Biochem.*, **88**, 1219 (1980).
10) J. Sunamoto, H. Kondo, and A. Yoshimatsu, *Biochim. Biophys. Acta*, **510**, 52 (1978).
11) P. Puchs, A. Parola, P. W. Bobbins, and E. R. Blout, *Proc. Natl. Acad. Sci. U.S.A.*, **72**, 3351 (1975).
12) W. P. Jencks, "Catalysis in Chemistry and Enzymology," McGraw-Hill, New York, N. Y. (1969), Chap. 7.
13) H. P. Gregor, J. Belle, and R. A. Marcus, *J. Am. Chem. Soc.*, **77**, 2713 (1955).
14) D. R. Robinson and W. P. Jencks, *J. Am. Chem. Soc.*, **87**, 2470 (1965).
15) C. Lee, *Biochemistry*, **10**, 4375 (1971).
16) A. Azzi, A. Fabbro, M. Santato, and P. L. Gherardini, *Eur. J. Biochem.*, **21**, 404 (1971).