CHEMISTRY LETTERS, pp. 1397-1400, 1981. © The Chemical Society of Japan 1981

SUBSTRATE ENTRAPMENT AND FORMATION OF pH GRADIENT IN AMMONIUM BILAYER VESICLES[1)]

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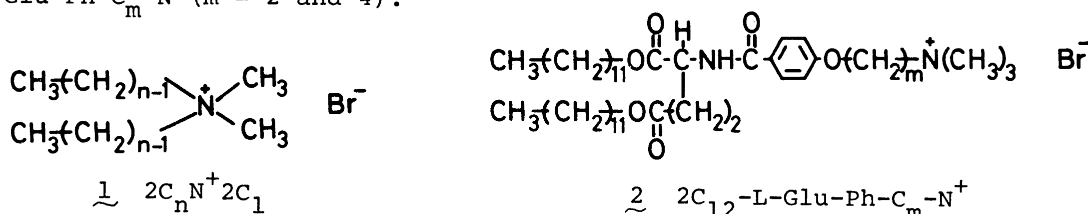
Trapping of water-soluble amines in the inner water core of ammonium bilayer vesicles was determined by reaction with fluorescamine, and the formation of pH gradient was confirmed by fluorescence quenching of membrane-bound riboflavin.

Compartmentalization is one of the fundamental characteristics of the bilayer membrane. Therefore, since formation of the synthetic bilayer vesicle was reported, trapping of various water-soluble substances in the inner water core has been examined by several groups.[3-6)] As the volume of the inner water core is very small relative to that of the outer aqueous phase, highly sensitive probes are required for the trapping experiment, and radio-active amino acids has been used frequently.

We describe in this letter the use of fluorescence probes for detection of trapped water-soluble substances and pH gradient across ammonium bilayer membranes. Bilayer-forming ammonium amphiphiles employed are $2C_nN^+2C_1Br^-$ (n = 16 and 18)[7)] and $2C_{12}$-L-Glu-Ph-C_m-N^+ (m = 2 and 4).[8,9)]



The first two ammonium salts(1) form lamellar aggregate predominantly. Aggregate morphologies of the latter two(2) are in sharp contrast as shown in Fig. 1. Amphiphile 2(m = 2) produces well-developed bilayer vesicles with diameter of 500 - 700 Å(Fig. 1a). Almost all vesicles seem to be double-walled. On the other hand, amphiphile 2(m = 4) gives fragmentary lamellae as in Fig. 1b. It is interesting that the structural difference in the spacer length by two CH_2 units causes this drastic change in the aggregate morphology.

Fluorescamine was used recently to label amino phospholipids in liposomes by Lee and Forte.[10)] We applied this technique to detection of trapped substances. D-Glucosamine and ethanolamine were employed as entrapment probes, because these neutral amines are conceivably retained only in the inner water core without binding to the membrane matrix.

In a typical trapping experiment, 0.4 mM of aqueous D-glucosamine was added to

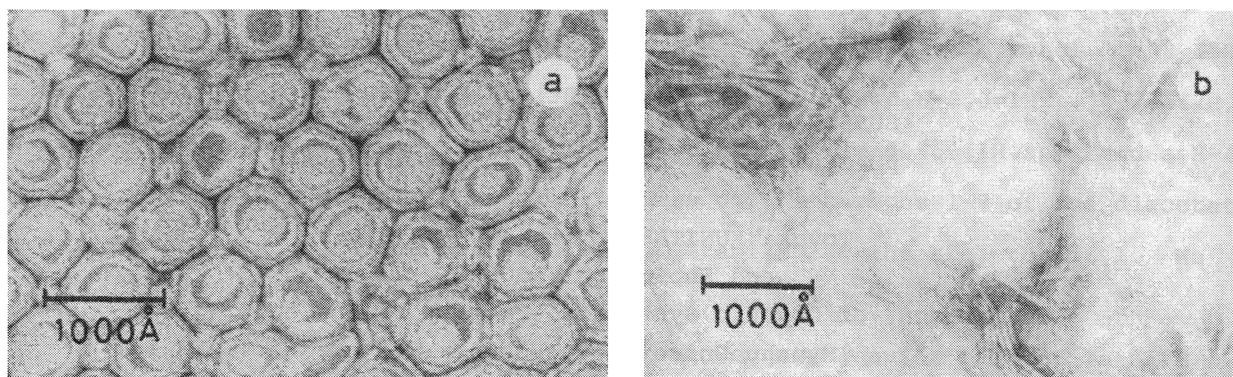
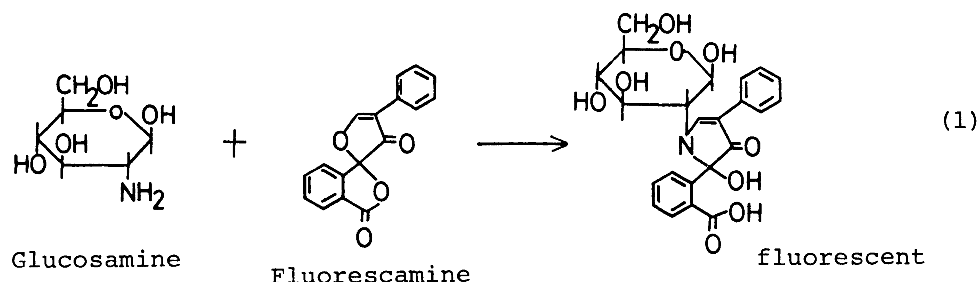


Fig. 1 Electron micrograph. Stained by uranyl acetate.
a: $2C_{12}$-L-Glu-Ph-C_2-N^+, initial magnification, × 50000
b: $2C_{12}$-L-Glu-Ph-C_4-N^+, initial magnification, × 60000

sonicated, aqueous solutions(pH 8.2 with 0.02 M borate buffer and μ = 0.01 with KCl) of dialkylammonium salts(20 mM) and sonicated for 20 - 30 s(Bransonic cell disruptor 185). The clear aqueous solution obtained was subjected to gel filtration at room temperature(ca. 18°C) using a Sephadex G-50 column(12 × 140 mm). Triton X-100(10 mM) was added to each fraction(1 ml) in order to destroy the bilayer membrane, and fluorescamine in acetone was injected through a microsyringe: [fluorescamine] = 0.8 - 1.0 mM. A 400-μl aliquot of this solution was then added to 1 ml of 5 mM citrate in ethanol, and the fluorescence intensity at 480 nm (excitation wavelength, 390 nm) was determined at 20°C. Excess fluorescamine was used to ensure the quantitative amine labeling and the observed fluorescence intensity is a direct measure of the amine present in each fraction.



(1)

Examples of the elution profile of glucosamine is shown in Fig. 2. Trapped glucosamine appears at the void volume(fraction Nos. 5 - 10) and untrapped glucosamine at fraction Nos. 15 - 30. The ratio of trapped glucosamine is 2.5 % in the case of $2C_{12}$-L-Glu-Ph-C_2-N^+ vesicles and negligibly small(∿0 %) for the $2C_{12}$-L-Glu-Ph-C_4-N^+ lamella. Similar trends were found for trapping of ethanolamine. The difference in trapping efficiency is clearly related to the presence and absence of the inner water core as inferred by electron microscopy. Aqueous aggregates of $2C_{18}N^+2C_1Br^-$ and $2C_{16}N^+2C_1Br^-$ gave trapping efficiency(glucosamine) of ca. 0.3 % and 0.5 %, respectively. The small trapping capability is consistent with the predominantly lamellar morphology found for these simple ammonium salts. Small amounts of glucosamine may be trapped in the concomitant vesicles or in the interlayer space of the lamella.

Kano and Fendler[11] showed that pyranine is a useful probe for detection of pH gradients across lecithin bilayers. Pyranine, however, is not suitable for ammonium bilayer vesicles, because it is strongly bound to the vesicle by the coulombic attraction. We instead used neutral riboflavin which loses proton at pH ∿10 and becomes non-fluorescent(Eq. 2).

In a typical riboflavin experiment, solutions of dialkylammonium salts(20 mM) and riboflavin(0.2 mM) in deionized water were cosonicated and subjected to gel filtration. The fluorescence intensity (ε_m) of membrane-bound riboflavin was measured at 522 nm with excitation wavelength of 380 nm. The fraction of bound riboflavin was 0.8 % with the $2C_{12}$-L-Glu-Ph-C_2N^+ vesicle and 0.5 % with the $2C_{18}N^+2C_1Br^-$ lamella.

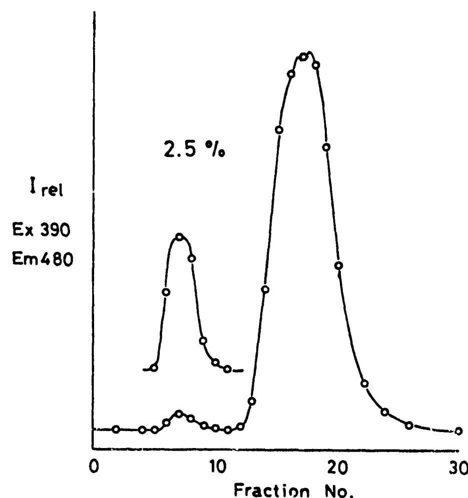
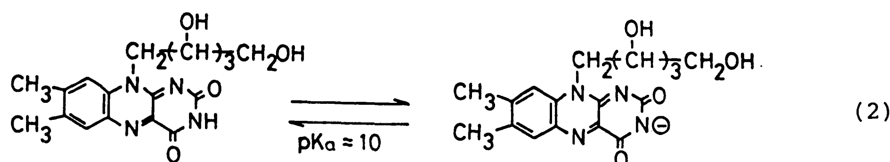


Fig. 2 Elution prophile of glucosamine in the presence of $2C_{12}$-L-Glu-Ph-C_2-N^+ vesicle.



(2)

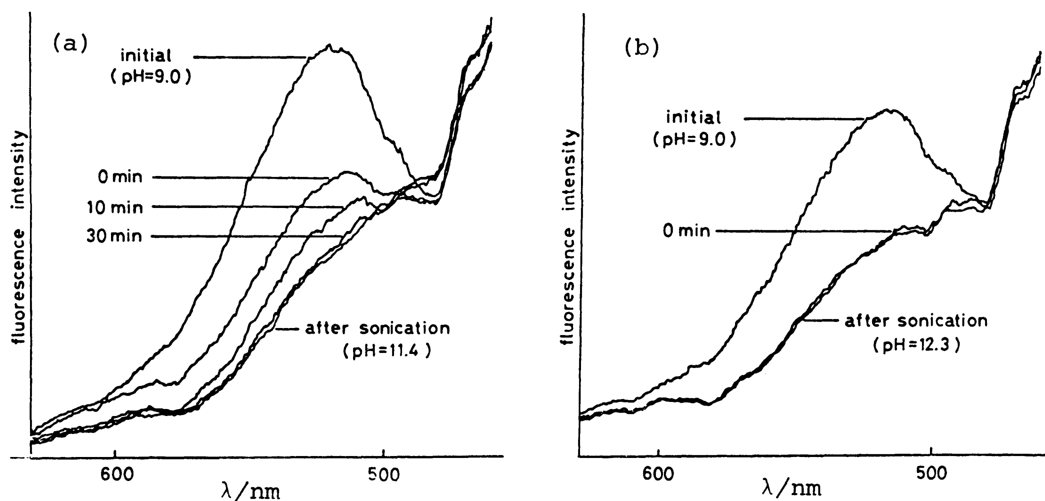
Riboflavin

The degree of depolarization of the fluorescense emission given by Eq. 3 was 0.018 and 0.35 ± 0.02 for aqueous and trapped(in $2C_{12}$-L-Glu-Ph-C_2-N^+) riboflavin, respectively. Insensitivity of trapping to the aggregate morphology and the enhanced P value suggests that the trapped riboflavin is bound to the membrane surface.

$$P = \frac{I_{\parallel\parallel} - I_{\parallel\perp}\cdot I_{\perp\parallel}/I_{\perp\perp}}{I_{\parallel\parallel} + I_{\parallel\perp}\cdot I_{\perp\parallel}/I_{\perp\perp}} \qquad (3)$$

Figure 3 illustrates the time course of fluorescence quenching. When pH of the vesicle solution was changed from 9.0 to 11.4 by addition of 1/200 N NaOH, the fluorescence intensity decreased instantaneously to one third of the original value(Fig. 3a). Apparently, ca. two thirds of riboflavin was bound to the outer surface of the vesicle and immediately deprotonated by alkali. The residual fluorescence gradually disappeared in 30 min. The final spectrum was little affected by sonication, indicating that permeation of OH^- into the inner water core was complete.

In contrast, when pH of the bulk phase was adjusted to 12.3 by addition of 1/50 N NaOH, riboflavin fluorescence was wholly quenched immediately. Permeation of OH^- is fast due to an increased pH gradient.



(a) 1/200 N NaOH added (b) 1/50 N NaOH added

Fig. 3 Fluorescence quenching of riboflavin by alkali (Eq. 2), 20°C.

In conclusion it is established that the morphological difference(vesicle vs, lamella) inferred by electron microscopy, corresponds well to the retention capability of the aggregate. It is remarkable that the ammonium membrane shows the barrier property against hydroxide ion. A variety of the trans-membrane function may be devised based on these results.

The authors are grateful to Prof. Motowo Takayanagi of Kyushu University for the use of an electron microscope(Hitachi, H-500). Mr. Hiroshi Fukushima kindly provided dialkyl glutamate amphiphiles.

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(Received July 24, 1981)