

Preconcentration of Catecholamines into Liposomes with Imposed pH Gradients

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The preconcentrations of epinephrine (EP) and norepinephrine (NE) into phosphatidylcholine liposomes with imposed pH gradient across the membrane were investigated by adding the catecholamine (CA) to the external medium of liposomes. To determine the CAs entrapped into liposomes, a simple method was also developed, based on the adsorption of CAs onto alumina and chemiluminescence detection. The uptake of the CAs into the liposomes was examined over a wide ΔpH range of 0–5 units (internal pH 5.0). The maximum uptakes of EP and NE were 70% and 88%, respectively, around an external pH of 9 (ΔpH 4), where zwitterionic species of EP and NE are dominant. The final concentrations of EP and NE in internal volumes were 34- and 43-fold greater than the initial concentrations of EP and NE, respectively. The uptake of the CAs was studied by using a mathematical model, which indicates that the uptake is contributed by the molar fraction of the zwitterion species in the external volume as well as the pH gradient. The uptake of EP was found to suffer from a steric hindrance due to a methyl group substituted for the hydrogen of the amino group. This study proves the capability of the liposomes for accumulation media of CAs.

Liposomes consisting of phospholipid bilayer have been studied as models for biomembranes and as drug carriers that deliver therapeutic agents in vivo. Drug carriers are based on the feature of liposomes capable of encapsulating compounds into their aqueous compartment. To date, the encapsulation of a variety of compounds into liposomes has been successfully used in cosmetics and food industries as well as clinical applications. Recently, liposomes encapsulating marker molecules or enzymes have been used analytically.[1–4] There are two approaches for the encapsulation of compounds into liposomes: First, compounds are included in the hydration buffer during liposome preparation. Second, compounds are added to the external phase of liposomes with a pH gradient (inside acidic) across the membrane. The latter technique has been applied to lipophilic amine drugs, such as chlorpromazine and lidocaine, which penetrate the liposomal membrane.[5] Moreover, the influence of the pH gradients on the permeability of drugs, lipids, and peptides has been extensively studied.[6]

Catecholamines (CAs), such as epinephrine (EP), norepinephrine (NE), and dopamine, were shown to be entrapped and accumulated into liposomes in response to ΔpH.[7] The uptake of those CAs into liposomes with a pH gradient of 3 units was 10–20-fold greater than that without pH gradients. This suggests the analytical capability of liposomes as media for the preconcentration of CAs. The mechanism of dopamine transport across the vesicular membrane was shown to follow acid-base equilibrium in internal and external volumes.[8] However, these studies were limited to a narrow range of pH gradient (0–3.6 units). Moreover, in these studies free CAs were chromatographically separated, and the uptake was determined by using radioactive CAs. These procedures were time-consuming and troublesome because of a long separation time and the handling of radioisotopes. Therefore, a simple method is needed to determine CAs entrapped into liposomes.

In this paper, we describe the uptake of EP and NE into liposomes over a wide ΔpH range of 0–5 units. For an uptake study, a simple method has been developed to determine CAs entrapped into liposomes, based on an alumina adsorption technique and a lucigenin-chemiluminescence (CL) detection that we previously reported.[9] The uptake of EP and NE was discussed in terms of the molar fraction of permeable species, a transmembrane pH gradient, and chemical structure.

Experimental

Materials. Epinephrine, norepinephrine, egg yolk phosphatidylcholine (PC), and cholesterol (Chol) were purchased from Wako Pure Chemical Industries (Japan). 10,10′-dimethyl-9,9′-biacridinium dinitrate (lucigenin, Luc) was obtained from Tokyo Chemical Industry (Japan). Triton X-100 was obtained form Nacalai Tesqdue (Japan). Alumina, ICN Alumina N-Super I was obtained from ICN Biomedicals GmbH. All solutions were prepared by using deionized (Milli-Q water-purification system, Millipore) distilled water. All chemicals were of reagent grade or better, and were used as received. All of the reagent concentrations shown in the figures and figure captions are the final concentrations.

Apparatus. Chemiluminescence measurements were made by using a homemade luminometer equipped with a photomultiplier and a recorder. An inductively coupled plasma atomic emission spectrometer ICPS-1000 Ⅳ (Shimadzu, Japan) was used to determine phospholipid phosphorus. Incubation was made with a shaker SA-31 (Yamato Scientific Co., Japan).

Pretreatment of Alumina. Approximately 50 g of alumina was suspended in 200 mL of 2 M HCl. The suspension was stirred for 30 min at 90 °C. The mixture was then allowed to cool and set-

tle, and the supernatant was withdrawn and discarded. The alumina was washed twice with 2 M HCl and resuspended in 200 mL of 2 M HCl for 30 min at 90 °C. The supernatant was again discarded and the alumina was washed repeatedly with water until the pH of the washings reached 3.5. Excess water was evaporated by heating the alumina for 3 h at 200 °C. The alumina was kept in tightly capped vials and stored in a desiccator until use.

Preparation of Liposomes. Liposomes were prepared by extrusion techniques.[10,11] A chloroform solution of PC or PC/cholesterol was added to a 100-mL round-bottom flask. Chloroform was removed by rotary evaporation at 30 °C for 1 h under reduced pressure and a stream of nitrogen gas forming a lipid film on the wall of the flask. The dried lipid film was hydrated in a 50 mM citrate–100 mM phosphate buffer at pH 5.0. A final lipid concentration of 10 mM in a PC system or 20 mM in a PC/Chol system was vortexed for 15 min at 25 °C to produce multilamellar vesicles (MLVs). The MLVs were extruded through polycarbonate filters with a pore size of 1000 nm. We subjected samples to 20 passes through a single filter. The filters were mounted in LiposoFast-Basic (Avestin Inc.) fitted with two 0.50 mL Hamilton syringes. The trapped volume of the liposomes was determined with calcein.[12] In order to establish the pH-differences across the vesicular membrane, the value of pH in the external medium was adjusted to pH 5–8 by adding a 1 M NaOH solution to the liposome suspensions.

Uptake of CAs and Determination of CAs Entrapped in Liposomes. A volume of 250 μL of 0.5 mM EP was added to a 12-mL suspension of liposomes with an imposed pH gradient across the membrane, and allowed to stir at 25 °C. The 2-mL aliquots of the mixture were withdrawn at time intervals and were mixed with 2.0 mL of water, 400 μL of 0.1 M EDTA, and the alumina. The mixture was stirred for 10 min after adjusting the pH value to 8.55 ± 0.1 with a NaOH solution, and unentrapped CA was then adsorbed on alumina. The mixture was transferred to micro tubes and centrifuged at 3000 rpm for 2 min. To a 500-μL aliquot of the supernatant in a glass cuvette, 500 μL of 10% Triton X-100 was added to destroy the membrane of liposomes. Then, the concentration of CA was determined by the lucigenin-CL method: A volume of 500 μL of 0.1 mM Luc and 500 μL of 0.1 M NaOH were injected simultaneously into the cuvette to initiate CL emission. The CL signal was measured with the luminometer and the signal-time profile was obtained. The CL intensity was defined as the maximum CL signal of the profile.

Results and Discussion

A Method for Determination of CAs Entrapped in Liposomes. We developed a simple method to separate the CAs entrapped in liposomes from the unentrapped CAs by using alumina, prior to an uptake study of CAs. Alumina has been used for a sample pretreatment for the assay of CAs in plasma, serum, and urine.[13–15] Catechol derivatives, such as epinephrine (EP) and norepinephrine (NE), specifically adsorb on alumina because of their two phenolic dihydroxy groups (the chemical structures are shown in Fig. 1). Therefore, the CAs entrapped into liposomes can be easily separated from free CAs by mixing the sample with alumina.

In order to optimize the procedure, EP was used as the model compound of CAs. The effect of the adsorption time on the amount of EP adsorbed on alumina was investigated. A 5-mL portion of a 20 μM EP solution containing 20 μM EDTA at pH 8.5 was added to 340 mg of alumina and shaken for 10, 20, and 30 min. The mixture was then centrifuged and EP in the supernatant was determined by the CL method. EP and NE almost fully adsorbed on the alumina in 10 min. The effect of the amount of alumina on the EP adsorption was investigated. A 5-mL portion of a 20 μM EP containing 20 μM EDTA at pH 8.5 was added to various amounts of alumina (150, 340, and 675 mg). The adsorption of 100% was obtained above 340 mg. To confirm that the alumina did not absorb the liposomes, the recovery of PC was examined by mixing a suspension (12 mL) of liposome exhibiting an external pH of 5–10 with 340 mg of alumina for 10 min. Centrifugation of the mixture was followed by the determination of PC in the supernatant by ICP spectrophotometry. For each external pH, the recovery of PC was 100%, which suggests that there is no loss of the liposomes entrapping CAs because of its adsorption onto the alumina. Finally, to compare the performance of the present method with a chromatographic method, separation was performed using a 1.0 × 30-cm Sephadex G-50 column. The liposomes and free EP were well separated in 2.5 h. This indicates that the chromatographic method was time-consuming because the separation of the liposomes and free EP was achieved within 20 min by the proposed method. Therefore, the proposed method based on alumina adsorption can be adopted for the separation of CA entrapped in the liposomes from free CA.

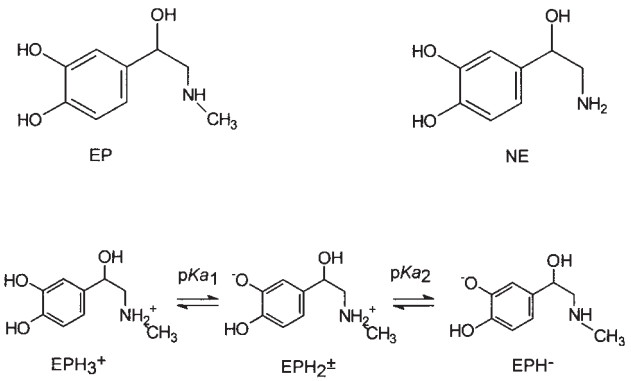

Fig. 1. Chemical structures of EP and NE, and acid dissociation equilibrium of EP in aqueous solution.

Uptake of CAs into Liposomes. We measured the uptake of EP and NE at time intervals of 20 min by using liposomes with imposed pH gradients in the range of 0–5 units (Fig. 2). The uptake was defined as the percentage of CAs (mol) entrapped into liposomes in CAs (mol) initially added into the external medium. The uptake of EP was linearly increased until 20 min, and reached the maximum at 60 min at all of the ΔpH values investigated. The uptake of NE reached the maximum at 80 min. These maximum values do not necessarily indicate that the uptake of the CAs had come to equilibrium, as described below. The amounts of NE entrapped at ΔpH of 0 and 1 units were not determined, because those amounts were lower than the limit of detection given by the proposed CL method, and the sensitivity for NE was 2-fold lower than that for EP. Incubation times of 60 and 80 min were chosen for the uptakes of EP and NE, respectively.

Figure 2 also shows that at the maximum 70% and 88% of EP and NE, respectively, were accumulated into the liposomes. The trapped volume of the liposomes used in the study was 2.1 μL/μmol lipid, which was determined by the cal-

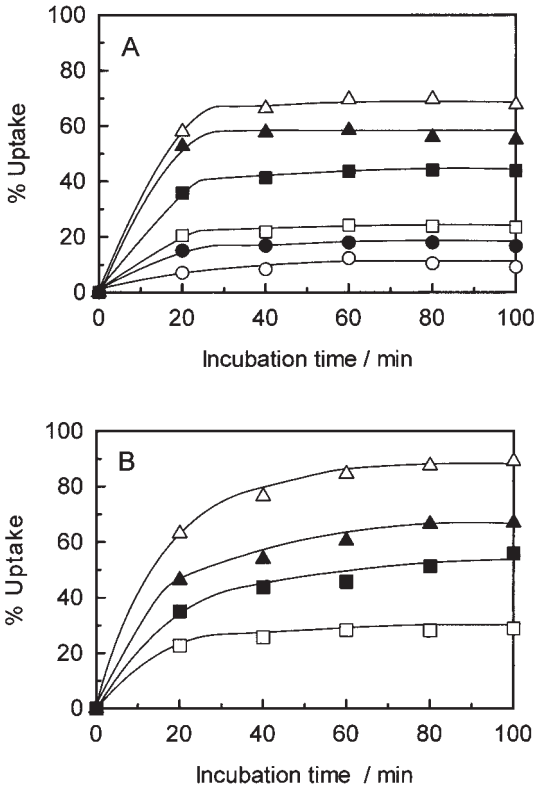

Fig. 2. Time course of uptake of EP (A) and NE (B) into liposomes exhibiting a various pH gradients (0 (○), 1 (●), 2 (□), 3 (■), 4 (△), and 5 (▲), internal pH 5.0). Initial external concentrations of EP and NE are 10 μM.

cein method. Thus, the final concentrations of these CAs in internal volumes were estimated by using the extent of the uptake and the trapped volume. Thus, the final concentrations of EP and NE were 34- and 43-fold, respectively, higher than the initial external concentrations. Moreover, these concentrations were 1.8 times higher than the internal concentrations at ΔpH 3. This demonstrates that the liposomes imposed appropriate pH gradients can be applied to the media for the preconcentration of CAs.

The uptake of CAs may be accompanied by a change in the pH gradient, because the penetrated neutral species is protonated to consume hydrogen ions in the internal volume.[7] The change in ΔpH may cause a decrease in the uptake. However, in the present study, the process of the uptake of EP does not involve a decay of the pH gradient, because the internal volume is well-buffered with high concentrations of citrate and phosphate. Furthermore, the pH gradient was found to be maintained by measuring the pH gradient with 9-aminoacridine.[16]

Effect of the Transmembrane pH Gradient on the CAs Uptake. The maximum uptake of EP and NE at each pH gradient is plotted in Fig. 3 according to the results shown in Fig. 2. The uptake of EP and NE increased with an increase in ΔpH, and reached the maximum value at around ΔpH 4. Above ΔpH of 4, the uptake decreased. These results show that the uptakes of EP and NE are dependent on the transmembrane pH gradient. Moreover, an investigation of the uptake of these CAs over a wide range of pH gradients reveals that the maximum uptake was observed at around ΔpH 4.

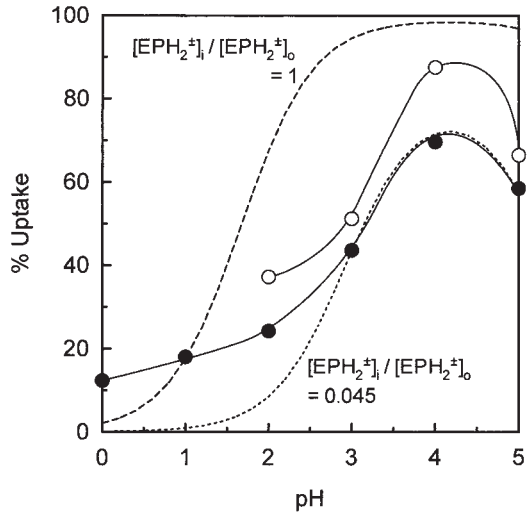

Fig. 3. Effect of transmembrane pH gradient on uptake of EP (●) and NE (○) into liposomes (internal pH 5.0). The dashed and dotted lines indicate the uptake curves of EP calculated from Eqs. 7–9 with $[EPH_2^{\pm}]_i/[EPH_2^{\pm}]_o$ of 1 and 0.045, respectively (internal pH 5.0).

In general, the transmembrane pH gradient-induced uptake of monoamine is presumed to be as follows. The neutral form (A) of monoamine penetrates the vesicular membrane, and the charged form (AH^+) cannot penetrate the membrane. Penetration of the neutral form of monoamine continues until the neutral amine concentration in the internal volume is equal to that in the external volume. Consequently, monoamine redistributes between the liposome internal volume and the external volume, depending on the pH gradient, as given by

$$\frac{[A]_i^T}{[A]_o^T} = \frac{[H^+]_i}{[H^+]_o} \quad (\text{for } [H^+]_i \text{ and } [H^+]_o \gg K_a), \qquad (1)$$

where subscripts i and o indicate the internal and external volume of the liposomes. However, Eq. 1 has a limitation for explaining that the EP uptake exhibits the maximum value. For describing the uptake of EP and NE over a wide range of the pH gradient, it should be taken into account that CAs have ionizable groups, such as hydroxy groups.

For a consideration of the EP uptake over a wide range of ΔpH, the molar fractions of EP were estimated as a function of pH according to the dissociation constants, pK_{a_1} (8.88) and pK_{a_2} (9.8), for the hydroxy group and amino group, respectively. As a result, the zwitterionic species, $EPH_2^{\pm}$, is dominant at around pH 9. This pH value corresponds to the external pH value (ΔpH of 4 units) that gave the maximum uptake of EP. The zwitterionic species as well as neutral species are membrane permeable.[17] Thus, these facts suggest that the zwitterionic species participates in the penetration of EP across the liposomal membrane. On the other hand, when the EP uptake was measured using liposomes, exhibiting that both the internal and external pHs were 9.0 (ΔpH 0), the uptake was 3%, though the uptake was 70% with liposomes exhibiting an internal pH of 5 and an external pH of 9 (ΔpH 4). This indicates that a high concentration of the zwitterionic species in the external volume can not necessarily result in a high level of uptake, though the zwitterionic is dominant in the external volume at pH 9. There-

fore, the acidic internal volume as well as the external molar fraction of the zwitterionic species would be essential for the pH gradient-induced EP uptake.

We further investigated the uptake of EP over the range of ΔpH 0–5 by a mathematical treatment of the uptake, as follows.

The total external and internal concentrations are given by

$$[EP]_i^T = [EPH_3^+]_i + [EPH_2^\pm]_i + [EPH^-]_i, \quad (2)$$

$$[EP]_o^T = [EPH_3^+]_o + [EPH_2^\pm]_o + [EPH^-]_o, \quad (3)$$

where subscripts i and o indicate the internal or external phase of the liposomes.

By introducing the dissociation constants, K_{a_1} and K_{a_2}, for the hydroxy group and the amino group, respectively, Eqs. 2 and 3 can be rearranged as follows:

$$[EP]_i^T = [EPH_2^\pm]_i \frac{[H^+]_i^2 + K_{a_1}[H^+]_i + K_{a_1}K_{a_2}}{K_{a_1}[H^+]_i}, \quad (4)$$

$$[EP]_o^T = [EPH_2^\pm]_o \frac{[H^+]_o^2 + K_{a_1}[H^+]_o + K_{a_1}K_{a_2}}{K_{a_1}[H^+]_o}, \quad (5)$$

where

$$K_{a_1} = \frac{[EPH_2^\pm][H^+]}{[EPH_3^+]}, \quad K_{a_2} = \frac{[EPH^-][H^+]}{[EPH_2^\pm]}.$$

The distribution ratio (D) of internal to external concentration of EP is given by

$$D = \frac{[EP]_i^T}{[EP]_o^T} \quad (6)$$

$$D = \frac{[EPH_2^\pm]_i}{[EPH_2^\pm]_o} \cdot \frac{[H^+]_o([H^+]_i^2 + K_{a_1}[H^+]_i + K_{a_1}K_{a_2})}{[H^+]_i([H^+]_o^2 + K_{a_1}[H^+]_o + K_{a_1}K_{a_2})}, \quad (7)$$

where we can assume that the internal concentration of the zwitterionic species, $EPH_2^\pm$, is equal to the external concentration in the uptake equilibrium ($[EPH_2^\pm]_i/[EPH_2^\pm]_o = 1$).

The uptake (u) of EP is given by

$$u = \frac{[EP]_i^T V_i}{[EP]_i^T V_i + [EP]_o^T V_o} \times 100\%. \quad (8)$$

Using Eq. 7, Eq. 8 can be expressed as

$$u = \frac{100Dt}{Dt + 1}, \quad (9)$$

where $t \approx V_i/V_o$.

The uptake curves calculated by using Eqs. 7–9 with a trapped volume (t) of 0.021 are shown in Fig. 3. The uptake curve with $[EPH_2^\pm]_i/[EPH_2^\pm]_o = 1$ shows a maximum value of 98% around ΔpH 4. The experimental results are lower than those expected from the calculation. However, the data calculated with $[EPH_2^\pm]_i/[EPH_2^\pm]_o = 0.045$ show agreement with the experimental data, though the disagreement was observed at ΔpH below 3. These facts suggest that the uptake of EP could not reach the state of equilibrium, and that the uptake rate of EP could be considerably low under these conditions. The disagreement at lower pHs may be attributable to a regeneration of the pH gradient in the process of the uptake measurement, in which the value of the external pH was readjusted to 8.5, as described in the experimental section.

The mechanism of the NE uptake in response to the pH gradient should be analogous to that of the EP uptake described above because the chemical structures and the pK_a values (pK_{a_1} = 8.82 and pK_{a_2} = 9.98) of NE are similar to those of EP. The experimental results for NE were roughly consistent with those calculated with $[NEH_2^\pm]_i/[NEH_2^\pm]_o = 0.075$. Therefore, the uptake of EP and NE is likely to be explained by the acid-base equilibrium in internal and external volumes by considering the uptake rate of these CAs.

Effect of the Structures of the CAs on the CAs Uptake and the Uptake Rate. The low uptake rates of EP and NE are presumably responsible for the membrane permeability to the CAs. In general, the membrane permeability is dependent on the incubation temperature, the chemical structure of the substance, the lipid composition, the liposome size, and the multiplex of lamella. Several studies demonstrated that the uptake efficiency of some compounds depended largely on the incubation temperature. For example, the maximum uptake of doxorubicin reached 100% at 60 °C, though the maximum uptake reached only 30% at 21 °C.[18] On the other hand, the uptake rate for EP and NE were relatively slower than that of lipophilic amines, such as 9-aminoacridine.[7] The slow uptake of these CAs may also result from a hindrance to transbilayer transport by hydroxyl groups of the CAs, because these hydroxyl groups are hydrated to have several water molecules around themselves.

The initial uptake rate of NE was greater than that of EP (Fig. 2). Moreover, the maximum amount of NE entrapped in the liposomes was greater than that of EP over the ΔpH range that we examined, as shown in Fig. 3. When the uptake is not in equilibrium, the efficiency of the uptake as well as the uptake rate is dependent on their membrane permeability to the CAs. Therefore, these facts indicate the difference in the membrane permeability between these CAs. This difference is attributable to the difference in the chemical structures: EP has a methyl

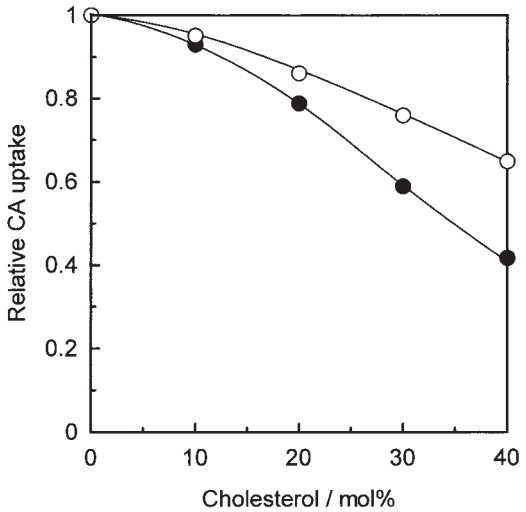

Fig. 4. Effect of cholesterol content on uptake of EP (●) and NE (○). The transmembrane pH gradient of 4 units (internal pH 5.0, external pH 9.0) was imposed on the liposomes. Initial external concentrations of EP and NE are 10 μM.

group substituted for a hydrogen atom of an amino group. As described above, the membrane permeability is related to the liposome lipid composition. In general, the increase in the content of cholesterol in the liposomal membrane reduces the membrane permeability because cholesterol hydrophobically interacts with phospholipid to reduce the membrane fluidity.[19,20] We then examined the uptake of EP and NE into liposomes prepared in various PC:cholesterol ratios. Figure 4 shows that the relative uptake of EP and NE decreased with increasing the cholesterol content. Evidently, the uptake of these CAs is related to the membrane permeability. The results were not likely in equilibrium though the time courses of the uptake showed apparent maximum values, similarly to Fig. 2. Figure 4 also shows that the relative uptake of EP remarkably decreases, compared with NE. When the cholesterol content increased up to 40%, the relative uptakes of EP and NE decreased by approximately 60 and 35%, respectively. These results indicate that EP is less permeable than NE. Therefore, the transport of EP across the liposomal membrane is hindered due to the methyl group.

Conclusion

The preconcentrations of EP and NE into liposomes were investigated over a wide range of transmembrane pH gradients (0–5 units, internal pH 5.0). In order to determine the amount of those CAs entrapped into liposomes, a simple method was also developed, based on the alumina adsorption and chemiluminescence detection of catecholamines. This method was successively applied to a study of the uptakes of EP and NE. The uptake was discussed in view of the pH gradient and the chemical structure of the CAs. A high level of preconcentration was achieved around ΔpH value of 4 through an uptake study over a wide range of the pH gradient. To describe this uptake, a mathematical model was used. The experimental results were roughly consistent with this model. This indicates that the uptake of EP and NE is contributed by the molar fraction of the zwitterion species, as well as the transmembrane pH gradient. A comparison of the EP and NE uptakes indicates that a methyl group of EP had a large influence on the transport across the liposomal membrane. The present study proves that liposomes with imposed pH gradients can be applied to the concentration system of CAs.

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