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LIPOSOMAL MEMBRANES

I. CHEMICAL DAMAGE OF LIPOSOMAL MEMBRANES WITH FUNCTIONAL DETERGENT

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Summary

The interaction and reaction between liposomal membrane and a functional detergent, *N*-hexadecyl-*N*-(imidazol-4-yl)methyl-*N*,*N*-dimethylammonium chloride hydroperchlorate (Im-I), have been investigated in conjunction with the leakage of bromothymol blue encapsulated as a marker in the bilayers of liposomes. Im-I carries an imidazole moiety and was expected to behave as a simple lipase model.

The reaction with Im-I significantly enhanced the leakage of bromothymol blue encapsulated in the egg lecithin and dipalmitoyl phosphatidylcholine liposomes. During the course of reaction with Im-I, the formation of acyl-imidazole intermediate was clearly identified, which was certainly connected with the bromothymol blue release. From various kinetic results on bromothymol blue release and acyl-imidazole formation, it has been suggested that the bromothymol blue release from liposomal bilayer may be caused by the local and instantaneous decomposition of lipids when Im-I penetrates into the bilayer. However, it has also been demonstrated that the immediate reconstruction of liposomes retains the barrier function to protect against the further release of bromothymol blue.

Introduction

Liposomes have been extensively investigated as biomembrane models. In addition to their abilities to mimic the function of barrier in membranes, liposomes are being developed as drug carriers. The drug delivery mechanism involves the intact entry of the liposome-encapsulated drug into the cell, where lipases or other factors cause the biodegradation of the membrane and release the drug from liposomal capsules [1—4].

Understanding of the interactions between lipids and additives, such as antigents [5,6], antibiotics [7—9], complements [10,11], or detergents [12], is an important problem in membrane biology. Investigation of the lytic properties of membranes and release of drugs from liposomal vesicles is desirable. Considering liposomes as the drug carrier, two features must be pointed out. One is the encapsulation of water-soluble drugs to pass through the hydrophobic cell membranes. The other is the penetration of water-insoluble materials into the hydrophobic bilayer of liposomes and delivery of them through the bloodstream to the target organ.

Previous investigations on liposome encapsulations have been mostly concerned with the water-soluble substrates using multicompartment liposomes. However, the multicompartment liposomes are less uniform and make good reproducibility of experimental data difficult to obtain. In this work, we used single compartment liposomes and a hydrophobic probe, bromothymol blue [13,14], entrapped in phosphatidylcholine bilayer.

N-Hexadecyl-*N*-(imidazol-4-yl)methyl-*N*,*N*-dimethylammonium chloride hydroperchlorate (Im-I) was used as a functional detergent since it carriers an imidazole moiety and is therefore, expected to behave as a simple lipase model [15,16]. The results obtained are discussed with particular attention to the leakage of bromothymol blue from liposomal bilayer and the lytic and/or self-repairing properties (fragility) of liposomal membranes.

Materials and Methods

Materials. Synthetic dipalmitoyl-DL-α-phosphatidylcholine was used as received from Sigma Chemical Company, St. Louis, Mo., or synthetized by deacylation and reacylation starting from egg yolk lecithin and purified according to the procedures described in refs. 17 and 18. No difference in the purity on thin-layer chromatography and in the melting point was observed by either group. Egg yolk lecithin was isolated and purified from egg yolk as described in refs. 19—21. Phospholipids were found to be pure on thin layer chromatography using a precoated Silica gel plate (Yamato replate 26) and chloroform/methanol/water (65 : 25 : 4, by vol.) as the eluant. Bromothymol blue and bromocresol green, reagent grade, were obtained from Nakarai Pure Chemicals Co. Ltd., Kyoto. Since the undissociated neutral form of dyes was quantitatively distributed into the organic phase during the solvent extraction, dyes were dissolved in dilute hydrochloric acid (pH 2), extracted with chloroform and obtained as the neutral form of dyes after removing chloroform. Im-I was synthesized according to the procedures described in refs. 22 and 23 with minor modifications. Because the hydrochloride was very hygroscopic [22,23], it was converted to the hydroperchlorate; m.p. 171°C. Found: C, 53.92: H, 9.62; N, 8.44. Calculated for $C_{22}H_{45}O_4N_3Cl_2$: C, 54.31; H, 9.32; N, 8.64%. δ([$^2H_6$]dimethylsulfoxide): 0.88 (3H, t, $CH_3\underline{CH_2}$-), 1.27 br (approx. 26H, CH_3-($\underline{CH_2}$)$_{13}$-CH_2-), 1.83 br (2H, m, -$\underline{CH_2}CH_2\overset{+}{N}\equiv$), 3.04 (6H, s, -($\underline{CH_3}$)$_2\overset{+}{N}$=), 3.33 (2H, t, -$CH_2\underline{CH_2}\overset{+}{N}\equiv$), 4.70 (2H, s, $\equiv\overset{+}{N}\underline{CH_2}$Im), 7.95 (1H, s, H(5) of imidazole ring), and 9.15 ppm (1H, s, (H(2) of imidazole ring). Nujol mull: ν_{C-H} (imidazole ring), 3120; $\nu_{\overset{+}{N}-H}$ (imidazole ring), 2720 and 2680; $\nu_{\overset{+}{N}-H}$ (alkyl), 2600; $\nu_{C=N}$, 1620; $\nu_{Cl-O(ClO_4^-)}$, 1100 and 1070; and δ_{C-H}, 830 cm^{-1}.

Other reagents were commercially obtained as analytical grade and used without further purification.

Preparation of liposomes. In order to obtain relatively well packed liposomes and to minimize the spontaneous release of marker from the soft vesicles [4], a given amount of cholesterol was used for all the liposome preparations in this work. Liposomes were prepared by evaporating 1.0 ml of chloroform solution containing phospholipid (0.79 μmol), cholesterol (0.52 μmol) and the marker (0.79 μmol) (in the molar ratio of 1.5 : 1.0 : 1.5, respectively) to dryness in a round-bottom flask using a rotary vacuum evaporator. Complete removal of volatile materials was accomplished by storing the flask in a vacuum desiccator overnight. The remaining thin film was dispersed by adding appropriate amounts of water, usually 2.0 ml, containing 0.1 M NaCl. The dispersion of phosphatidylcholines was carried out at a temperature about 10°C higher than the phase transition temperature of the used phospholipid, by shaking on a Vortex mixer with glass beads until foaming ceased. The resulting milky suspension consisted mostly of multicompartment liposomes [24—27]. Single compartment liposomes were prepared from the multicompartment liposomes by ultrasonication under nitrogen atmosphere [25—27] using a Tomy UR-200P probe-type sonifier at 25 W for 15 min at 1 min intervals. The relatively cleared, sonified solution of liposomes was passed through a Sephadex G-50 column [2,3]. Liposome-encapsulated markers appeared in the void volume and were recognized by their turbidity. Fig. 1 shows the gel-filtration pattern of free and lipsome-encapsulated bromothymol blue using egg lecithin and dipalmitoyl phosphatidylcholine liposomes, respectively. For optical purposes, turbidity was eliminated by ultrafiltration using a Sartrius Membrane filter SM16315, equipped with a filter SM11309 (0.1 μm pore size), SM11310 (0.05 μm), or SM11311 (0.01 μm). In a few cases, the aggregates of vesicles were separated out by ultracentrifugation on a Tominaga Works, Model S-62 ultracentrifuge at 2°C and 15 000 rev./min (33 000 $\times g$) for 20 min. After the gel filtration, both the liposome-encapsulated and free markers were collected and each was determined spectrophotometrically: bromocresol blue, $\epsilon_{617} = 4.0 \cdot 10^4\ M^{-1} \cdot cm^{-1}$ and bromothymol blue, $\epsilon_{617} = 7.3 \cdot 10^3\ M^{-1} \cdot cm^{-1}$, at pH 7. For this purpose liposomes were completely degraded by heating for 30 min at 80—90°C and higher pH on a steam bath prior to the determination of marker concentration. Throughout the present investigation no ionic substances, such as stearylamino or dicetylphosphate, were added, so the surface charge of the membranes could not be controlled. The surface charge was, then, kept neutral in all runs.

Determination of liposome concentration. Using optically clear solution, the liposome concentration was spectrophotometrically obtained at 210 nm [28]. Calibration curves were prepared on the basis of phosphorus content of phosphatidylcholines as determined by Allen's procedures [29].

In most runs, the formation of single compartment liposomes was visualized by negatively staining with potassium phosphotungstate in the JEOL JEM-100V electron microscope. No indications of the presence of multicompartment vesicles were obtained by the electron microscopy.

Adjustment of pH environment of liposomes. Scarpa and de Gier [30] have demonstrated that, even when liposomes were suspended in media different

from that in which they are formed, good pH equilibrations were retained with a pH gradient (ΔpH) of about 0.1 pH units between the interior and exterior of liposomes. Very recently, however, Kaback and co-workers [31,32] have reported that, despite variation in the external pH values, internal pH value remains essentially constant in *Escherichia coli* membrane vesicles. Therefore, first, liposomal membrane vesicles were dispersed, sonified, and gel filtered in 0.1 M NaCl without buffer solution. Thereafter, the liposome solution was resuspended in 55 mM buffer at a given pH containing 0.1 M NaCl. By this method, a transmembrane pH gradient (interior unbuffered neutral) was retained *. Even if bromothymol blue leaked into the interior pool of vesicles from the hydrophobic bilayer, the spectroscopic appearance due to the dissociated species of dye would be negligible.

Kinetics of bromothymol blue release induced by Im-I. 1.0 ml of optically clear solution of liposome-encapsulated bromothymol blue and 2.0 ml of buffer containing 0.1 M NaCl were placed in a cuvette cell and preincubated for 10 min at a given temperature. The bromothymol blue release induced by the addition of Im-I was followed by monitoring the increase of intensity at 617 nm on a Hitachi 124 recording spectrophotometer equipped with a thermoregulated cell holder. The percentage of bromothymol blue release was calculated by Eqn. 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 buffer solution was added to the liposome solution, respectively. A_{total} stands for the absorbance of total amount of liposome-encapsulated marker. The A_{total} value was determined after the complete degradation of liposomes by the method described previously.

$$\frac{A_{total} - A_t}{A_{total} - A_0} \times 100 = \% \text{ bromothymol blue-release} \quad (1)$$

Maximum % release of bromothymol blue was obtained from the plateau region in the kinetics, usually after incubation for 30 min.

Results and Discussion

1. Entrapment of marker in liposomes. Both bromothymol blue and bromocresol green were more effectively entrapped in egg lecithin liposomes than in dipalmitoyl phosphatidylcholine liposomes (Fig. 1). Assuming that each liposome contains approximately 3000 phospholipid molecules [25], we estimate the concentration of single compartment liposomes to be about $1-8 \cdot 10^{-8}$ M by assaying phosphorus contents and using the appropriate spectroscopic calibration. These concentrations reveal that each egg lecithin vesicle contains 700—1000 molecules of bromothymol blue, while dipalmitoyl phosphatidylcholine vesicles contain 50—150 molecules of bromothymol blue.

2. Microenvironment of marker in liposomal membrane. Different kinds of probes, such as dyes [13,14], fluorescent probes [33,34], or spin-labelled com-

* K. Kano and J.H. Fendler have very recently demonstrated the retention of the pH gradient in liposomes using a fluorescent probe, pyranine, as a sensitive pH indicator [48].

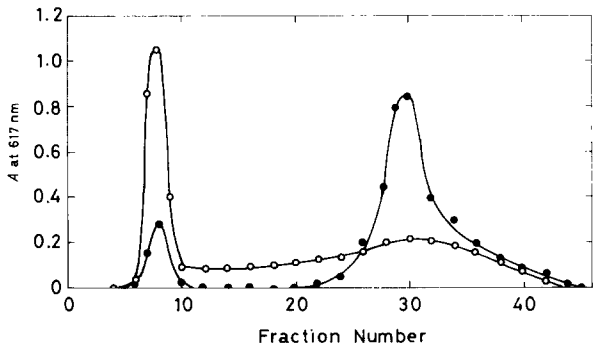


Fig. 1. Absorbance of bromothymol blue at 617 nm of 3 ml eluent fractions from a Sephadex G-50 column (1.8 X 35 cm) in 0.1 M NaCl for vesicles of egg lecithin (○) and dipalmitoyl phosphatidylcholine (●), also containing 0.1 M NaCl.

pounds [35] have been extensively utilized to obtain information about the microenvironment in liposomes and biomembranes. A knowledge of location of marker in liposomal vesicles is required prior to investigating the penetration into or leakage from the liposomal bilayer. Ultraviolet and visible spectra of bromothymol blue and bromocresol green were examined in different solvents to prepare a measure of the apparent polarity around the probe in the bilayer. Fig. 2 includes plots of absorption maxima and log ϵ_{max} of bromothymol blue against the microscopic solvent polarity parameter E_T (30) [36]. These data indicate the apparent environment of bromothymol blue in single compartment liposomes to be roughly correspondent to that in ethanol or methanol. For bromocresol green, a complete agreement was obtained (not shown). There is another point to note: only in aqueous solution did these

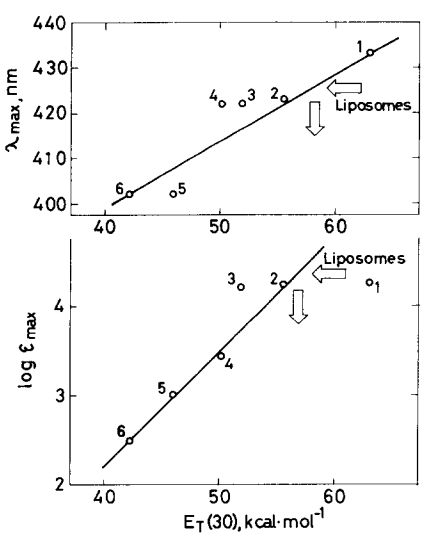


Fig. 2. Plots of absorption maxima of bromothymol blue in different solvents and log ϵ at the corresponding absorption maxima versus solvent polarity parameter E_T(30). Blank arrow indicates the polarity of microenvironment around probes encapsulated in liposomes. Solvents: 1, water; 2, methanol; 3, ethanol; 4, *n*-butanol; 5, acetonitrile; 6, acetone.

probes have two absorption maxima, approx. 430—440 and 617 nm, which are assigned to the undissociated and dissociated species of dye, respectively. In organic solvents and in liposomes, however, no absorption due to the dissociated species (617 nm) appeared. This means that these dyes are not in the interior water pool of the vesicles, but are located in liposomal bilayer close to the surface.

3. The Im-I induced leakage of bromothymol blue located in the liposomal bilayer. When Im-I was added prior to the thin film formation by mixing with phosphatidylcholine in chloroform, the amount of single compartment liposomes, which were isolated by gel filtration, significantly decreased as compared with those prepared in the absence of Im-I. Moreover, two additonal components, which would be lysates of dipalmitoyl phosphatidylcholine with Im-I, were detected during the gel chromatography. This result tempted us to investigate the leakage to the exterior of hydrophobic probe located in the liposomal bilayer upon the chemical damage of liposomal membranes with the functional detergent.

The functional detergent, Im-I, carrying an imidazole moiety, would be expected to behave as a simple lipase model, since pancreatic lipase has recently been reported to very probably be a serine-histidine enzyme [15,16]. On the other hand, as described in Materials and Methods, the undissociated neutral form of bromothymol blue was completely located in the liposomal bilayer when it was encapsulated under unbuffered neutral condition.

Under conditions where the spontaneous release of bromothymol blue was relatively slow or depressed, the addition of Im-I to the exterior of liposomes much enhanced the leakage of bromothymol blue from the liposomal bilayer. Both the initial velocity and maximum of bromothymol blue release were related to the external pH, the Im-I concentration (solid circles in Fig. 3), and the incubation temperature (open circles in Fig. 4). Along with the bromothymol blue release, a new absorption band appeared at 243.5 nm during the reaction of liposomes with Im-I, as seen in Fig. 5b. This band must be assigned to the acyl-Im-I on the basis of the spectroscopic consistency between the liposomes sensitized with Im-I and Im-I acetylated with acetic anhydride [22]. During the course of reaction, both bromothymol blue release and acyl-Im-I formation proceeded according to the first-order kinetics with rate constants of $1.9 \pm 0.1 \cdot 10^{-3} \cdot s^{-1}$ and $4.1 \pm 0.5 \cdot 10^{-3} \cdot s^{-1}$, respectively, at external pH 6.98 and 25°C. However, it should be noted that only a limited amount of the marker, not all, is released in the subsequent process.

Judging from these results, it is evident that both processes are closely related to each other, as seen in Fig. 3, and consequently, the enhanced leakage of the marker must be caused by the chemical damage of liposomal membranes. The acyl-cleavage of phosphatidylcholines will give lysophosphatidylcholine and/or 2-acylglycerol-3-phosphorylcholine. From the thin layer chromatography investigation on the reaction mixture of egg lecithin liposomes with Im-I, the decomposed product was almost all egg yolk lysolecithin [37,38]. Under the present reaction conditions, the amount of lysophosphatidylcholines formed was not so great and most of them should be reorganized in the liposomes [12]. Clearly, from much previous evidence, the imidazole group is, in general, not necessarily a good nucleophile toward the ester, bear-

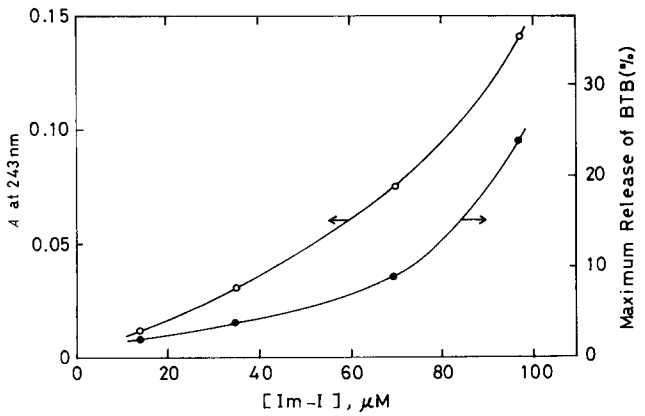


Fig. 3. The correlation between the bromothymol blue (BTB) release (●) and the acyl-Im-I formation (○) during the reaction with different concentrations of Im-I at pH 7.4 (external), 25.0 ± 0.1°C. The concentrations of egg lecithin liposomes and entrapped bromothymol blue were 70 nM and 54 μM, respectively.

ing a poor leaving group. Nevertheless, the fact that in considerable hydrophobic environment, as in the liposomal bilayer, even the imidazole group can act as a good nucleophile is of much interest and may be the first finding, besides cases supporting the ester, bearing a good leaving group such as the *p*-nitrophenyl group [22,23,39]. It is also of interest that the further deacylation process of the acyl-Im-I was completely depressed, as seen in Fig. 6. This may mean that hydroxide ions in the exterior bulk solution are not able to pene-

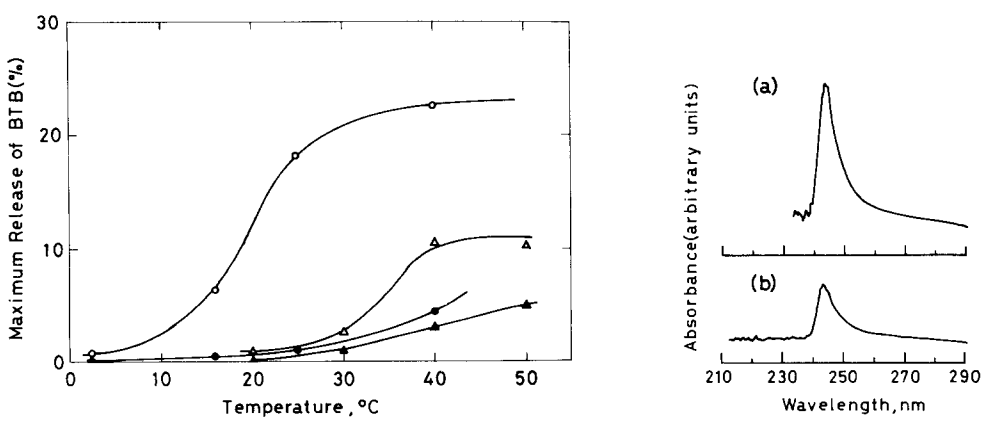


Fig. 4. The effect of incubation temperature on the Im-I induced releases of bromothymol blue (BTB) from egg lecithin liposomes (○———○) and dipalmitoyl phosphatidylcholine liposomes (△———△) at pH 7.4 (external). Controlled (spontaneous) releases from egg lecithin (●———●) and dipalmitoyl phosphatidylcholine liposomes (▲———▲) were also measured under the same conditions. In the case of egg lecithin liposomes, 70 nM liposomes encapsulating 56 μM bromothymol blue were reacted with 69.7 μM Im-I. In the dipalmitoyl phosphatidylcholine/bromothymol blue system: 10 nM liposomes containing 1.8 μM bromothymol blue with 69.7 μM Im-I.

Fig. 5. The spectroscopic consistency between the acetylated Im-I with acetic anhydride (a) and reacted liposomes with Im-I (b). (a), Im-I (10 mg) was acylated with 0.5 ml of acetic anhydride in the presence of 0.2 ml of triethylamine at 55 ± 5°C for 1 h. For the ultraviolet measurement, the reaction mixture was diluted with methanol to 5 ml. (b), Egg lecithin liposomes (70 nM) were incubated in the cuvette with Im-I (69.7 μM) at 40°C and pH 6.9 for 30 min.

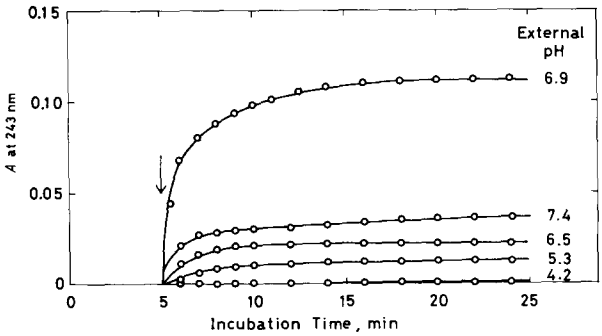


Fig. 6. The kinetics of acyl-Im-I formation during the reaction of egg lecithin liposomes (70 nM) with Im-I (69.7 μM) at 25°C by monitoring the absorbance at 243 nm. The arrow indicates the time at which Im-I was injected into the liposome solution.

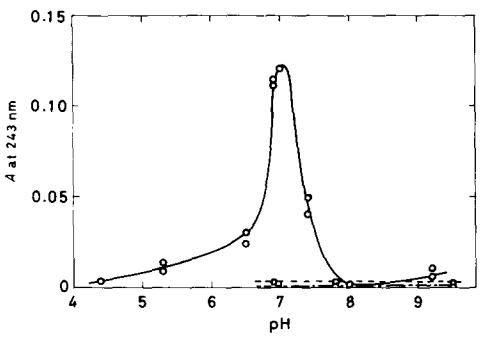


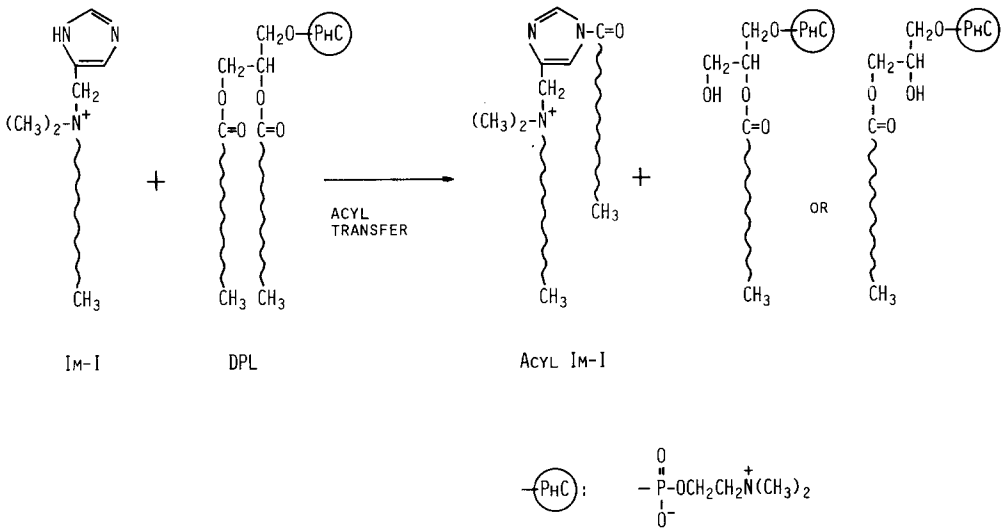
Fig. 7. The pH-rate profile for the acyl-intermediate formations. Egg lecithin liposomes (70 nM) were reacted with Im-I (69.7 μM, ○———○), imidazole (67.7 μM, □- - - - -□), and histidine (69.7 μM, △— · —△) at 25°C.

trate into the hydrophobic bilayer and deacylate the acyl-imidazole. Fig. 7 shows the pH-rate profile for the acyl-Im-I formation. One possibility for this bell shaped pH profile may be interpreted in terms of electrostatic repulsion brought about by the formal charge on the imidazole moiety. The protonated species of Im-I is predominant at pH lower than 7 *, while above pH 8.5 the contribution of the imidazoyl anion may be expected to become significant, as observed previously [40]. Ionic species must be unfavorable to interact with the neutral liposomal surfaces. The enhancement of leakage of marker brought about by Im-I should be primarily ascribed to the hydrophobic interaction between Im-I and liposomes, since imidazole and histidine failed to provide even the slightest evidence of forming the acyl-imidazole intermediate (Fig. 7).

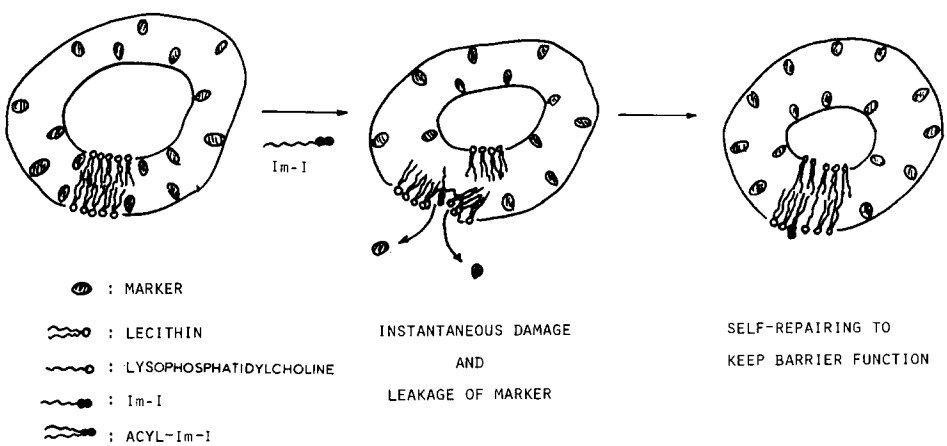
Considering the fact that the chemical damage of membranes is not steady, the instantaneous and local damage may be promptly self-repaired by rapid reaggregation of the acyl-Im-I and lysophosphatidylcholine to prevent further damage to the liposomal membrane and leakage of marker.

4. The effect of temperature on the enhanced leakage with Im-I. It is well recognized that dipalmitoyl phosphatidylcholine shows a higher phase transi-

* The imidazol ring of Im-I has been assumed to be entirely in the neutral form at pH 7.2 [23].



Scheme 1. Acyl transfer reaction.



Scheme 2. Schematic representation of mechanism for chemical damage of liposomes and leakage of markers.

tion temperature (about 41°C [41]) than egg lecithin (approx. —7 to —15°C [42]). On the other hand, cholesterol abolishes the phase transition and affords the fluidizing and condensing effects on the liposomes [43]. Certainly there was not so specific a difference between egg lecithin and dipalmitoyl phosphatidylcholine liposomes in the spontaneous release of bromothymol blue without Im-I (Fig. 4). When egg and dipalmitoyl phosphatidylcholine liposomes containing bromothymol blue were incubated with a given amount of Im-I at various temperatures for 30 min, however, the bromothymol blue releases were promoted at lower temperatures compared to those of the spontaneous release. Temperatures when the significant permeability appeared are not

necessarily related with their specific phase transition temperature of phosphatidylcholines. The temperature effect on the bromothymol blue release may reflect the differences in the tightness of the molecular packing between both liposomes. Another possibility for the Im-I-induced leakage of bromothymol blue is, therefore, the function of phase separation with Im-I, as seen in the increased leakage of glucose from liposomes treated with Triton X-100 or lysophosphatidylcholine [44—47]. However, from the evidence that cetyltrimethylammonium bromide did not provide any significant effect on the release (not shown), the elucidation in terms of the phase separation seems to be unsuitable for the present system. To release liposome-encapsulated drugs or markers, the chemical damage of liposomal membranes seems to be more effective than the change in the physical properties of lipids.

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