[Chem. Pharm. Bull. 33(1) 308–312 (1985)]

Chloride Leakage Measurement for the Evaluation of the Plasma-Induced Instability of Liposomal Membrane

SUMIE YOSHIOKA

National Institute of Hygienic Sciences, 1–18–1, Kamiyoga, Setagaya-ku, Tokyo 158, Japan

(Received April 19, 1984)

The plasma-induced instability of reverse-phase evaporation vesicles (REV) was successfully evaluated by measuring chloride leakage. The chloride efflux could be followed by using a silver chloride electrode without interference from coexisting substances such as liposomal lipid, plasma or bovine serum albumin (BSA). The permeability of REV was increased by incubation with human plasma or BSA; the effect of plasma was estimated to be approximately 50 times larger than that of a corresponding amount of albumin. The difference of protein–lipid interaction in the cases of plasma and isolated albumin is discussed on the basis of the leakage pattern and the activation energy of the permeation process.

Keywords——liposome; permeability; chloride leakage; reverse-phase evaporation vesicle; ion selective electrode; human plasma; bovin serum albumin

The stability of liposomes in the circulation is of great interest when they are applied as intravenous drug carriers. Liposomes are known to show increased permeability and to release entrapped solutes rapidly when they interact with plasma or serum.[1,2] Such plasma-induced instability of liposomes has been studied with small unilamellar or large multilamellar liposomes, and suggested to be due to the interactions of phosphatidylcholine with high density lipoproteins (HDL).[3–7]

The kinetic aspects of the solute leakage from liposomes in plasma should have significant implications for the effectiveness of liposomes as carriers.[8] Relatively little attention, however, has been given to the kinetics of solute release from liposomes enhanced by plasma, mainly because of the difficulty of continuous quantitative measurement.

The present paper is concerned with the kinetics of the chloride leakage from reverse-phase evaporation vesicles (REV)[9] induced by human plasma and bovine serum albumin (BSA) in connection with an evaluation of the membrane instability and of the preferred characteristics of liposomes as drug carriers. Chloride was chosen as a marker entrapped in liposomes because the mechanism of its permeation through bilayer membranes has been well studied,[10–14], and its efflux could be followed by using a silver chloride electrode to obtain kinetic data.

Experimental

Materials——Egg phosphatidylcholine (PC) and bovine serum albumin were purchased from Sigma Chemical Co. (St. Louis, U.S.A.). All other chemicals were of reagent grade. Phosphate buffer, pH 7.4, contained 136 mM $NaNO_3$, 3.2 mM KH_2PO_4 and 14.7 mM Na_2HPO_4. Human plasma was obtained by centrifuging pooled blood (Kokuritsu Shoni Hospital, Tokyo). Stock plasma was dialyzed at 4 °C against 100 volumes of buffered solution, which was changed 3 times over the subsequent 24 h period. BSA as received was dissolved in phosphate buffer (120 mg/ml stock solution), and dialyzed in the same way as stock plasma.

Preparation of Liposomes——Liposomes were prepared according to the method described by Szoka and Papahadjopoulos.[9] PC (131 μmol) was dissolved in 6.6 ml of diethylether and dispersed in 2.2 ml of 136 mM NaCl

solution buffered with 3.2 mM KH_2PO_4 and 14.7 mM Na_2HPO_4 to pH 7.4. Sonication was conducted at 4 °C for 3 min under nitrogen with a probe-type sonicator (Ohtake Ind., Ltd, 20 kHz, 150 W). The organic solvent was then removed under reduced pressure at 15 °C. After addition of 5 ml of buffer followed by filtration through a 0.6 μm pore-size polycarbonate membrane (Nuclepore Co.), liposomes were separated from non-entrapped chloride on a Sephadex G-50 column at 5 °C with phosphate-buffered solution as the eluant. The liposome-containing fractions (20 ml) were pooled as a stock liposomal suspension. The average diameter of the liposomes prepared as described above was determined to be 0.2 μm from negatively stained electron micrograms.[15] (The numbers of liposomes assigned to each size interval were 53 (<0.1 μm), 149 (0.1—0.2 μm), 91 (0.2—0.3 μm) and 6 (>0.3 μm).) The mean percentage of chloride entrapment was 25.0±1.8% (mean±S.D., $n=10$).

Leakage Studies——A 10 ml aliquot of stock liposomal solution was added to 10 ml of phosphate buffer, BSA solution (10—120 mg/ml) or human plasma solution (2—100%), to yield a final lipid concentration of 2.6 μmol per ml. Liposomes were then incubated under a nitrogen atmosphere at 15, 25, 37 or 45 °C in a 50 ml dual beaker with a magnetic stirrer. At appropriate intervals, a silver chloride electrode (Toa Denppa CL-125B) was immersed in the sample solution to determine chloride released from the liposomes. At the end of each run the total amount of chloride entrapped in liposomes was determined by addition of Triton X-100 (2% final concentration). The surfactant had no effect on the measurement of the electromotive force (emf).

Results and Discussion

A silver chloride electrode could be successfully employed for the determination of chloride released from liposomes even in the presence of liposomes, human plasma and BSA. Figure 1 shows the relationship between the emf and the chloride concentration. No interfering effect of these substances on the emf was observed.

The mechanism of chloride permeation through bilayer membranes has been well studied; the molecular form of HCl[11] or a phospholipid–HCl complex[12,14] is likely to permeate in association with a transbilayer "flip–flop" of phospholipid. On the basis of the

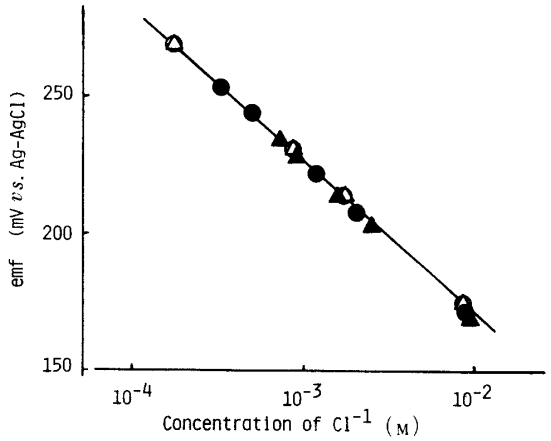

Fig. 1. The Relationship between emf of the Silver Chloride Electrode and Concentration of Cl^{-1} at 37 °C

○, standard soln. of Cl^{-1}; △, Cl^{-1} soln. containing liposomal lipid (2.6 μmol/ml); ●, Cl^{-1} soln. containing liposomal lipid and BSA (40 mg/ml); ▲, Cl^{-1} soln. containing liposomal lipid and plasma (50%).

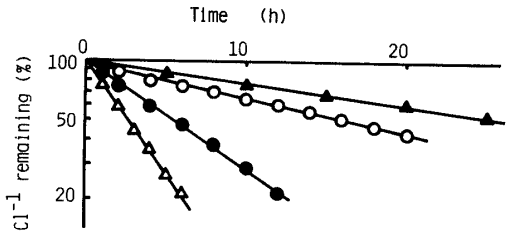

Fig. 2. The Effect of Temperature on Chloride Leakage from REV in Buffer Solution

▲, 15 °C; ○, 25 °C; ●, 37 °C; △, 45 °C. Liposomal lipid: 2.6 μmol/ml.

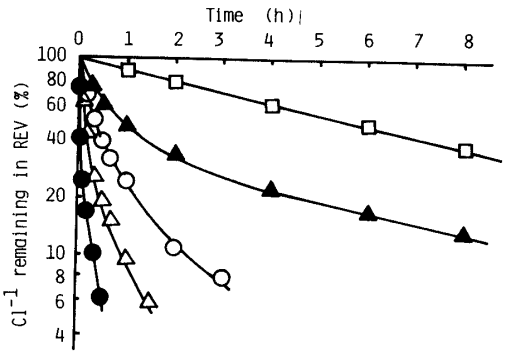

Fig. 3. The Effect of Plasma on Chloride Leakage from REV

Plasma concentration: □, 0; ▲, 1; ○, 2.5; △, 10; ●, 50%. Temperature: 37 °C. Liposomal lipid: 2.6 μmol/ml.

fact that the chloride permeation is electrically silent[14] and relatively fast, it should be an excellent measure to evaluate the stability of the liposomal membrane in various media. For the studies on the chloride permeability, isotopic chloride has commonly been used as a marker solute. The chloride leakage, however, can be more easily measured by using a silver chloride electrode, without separation of chloride from the liposomal fraction.

The leakage of chloride from liposomes in the buffer solution followed first-order kinetics with respect to remaining ion in the vesicles (Fig. 2). On the other hand, in the presence of plasma, the chloride leakage was remarkably enhanced in the early stage with increasing plasma concentration, followed by a retarded leakage (Fig. 3). Such a biphasic phenomenon is consistent with the non-linear kinetics reported for the sucrose release from multilamellar liposomes in plasma, where a rearrangement of membrane lipids and adsorbed proteins is suggested to cause considerable release retardation after an initial rapid rise in the liposomal membrane permeability.[8]

The chloride leakage from REV, however, proceeded in a manner parallel to the spontaneous leakage after the initial enhancement caused by plasma (Fig. 3). This suggests that the leakage enhancement by plasma proteins, probably HDL, is due to a temporary disorder of the bilayer structure and subsequently the rearrangement of lipid molecules with proteins results in the restoration of the barrier function of the bilayer. It has also been proposed that the enhanced leakage of entrapped solute is due to the irreversible extraction of lipid molecules from liposomes and the formation of non-liposomal water-soluble lipid–protein complexes.[3,16,17] However, this would not be relevant to the present case because the turbidity (optical density at 500 nm) remained unchanged during the leakage (data not shown).

The enhancing effect of plasma on the liposomal membrane permeability increased with increasing plasma concentration, and a tendency to saturate was observed, as shown in Fig. 4, where the initial rate is plotted against plasma concentration. The activation energy of the initial leakage rate accelerated by plasma (diluted to 50%) was estimated from the temperature dependency to be 20.5 kcal/mol deg (Fig. 5), which is higher than that of the spontaneous release rate (16.7 kcal/mol deg).

It has been reported that BSA can act as a modifier of the permeability of bilayers.[2-4,18] The chloride leakage in the presence of BSA was accelerated with increasing protein

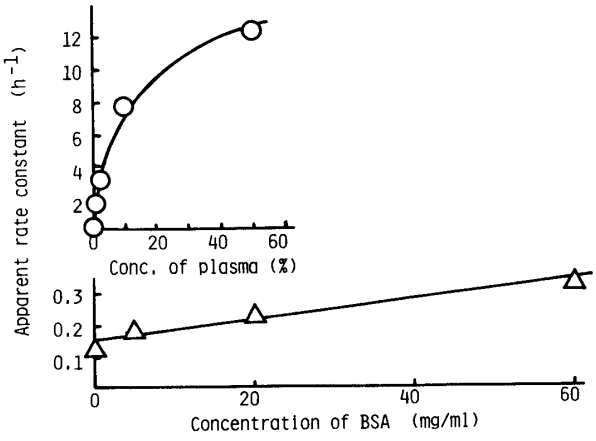

Fig. 4. The Effect of Plasma and BSA on the Rate Constant of Chloride Leakage

○, plasma (the initial rate constant was estimated by extrapolation to zero time); △, BSA.

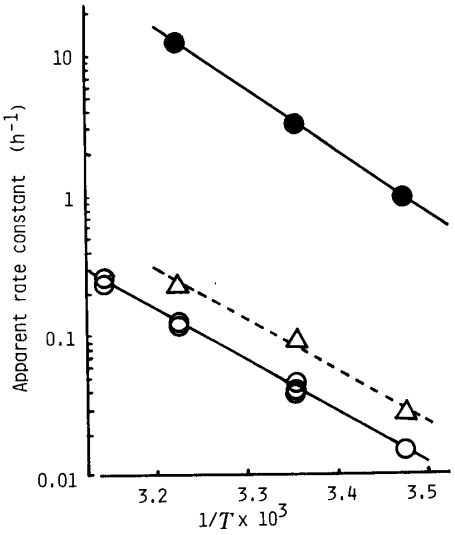

Fig. 5. Arrhenius Plots of the Rate Constant of Chloride Leakage

○, buffer solution; △, BSA solution (20 mg/ml); ●, plasma (50%).

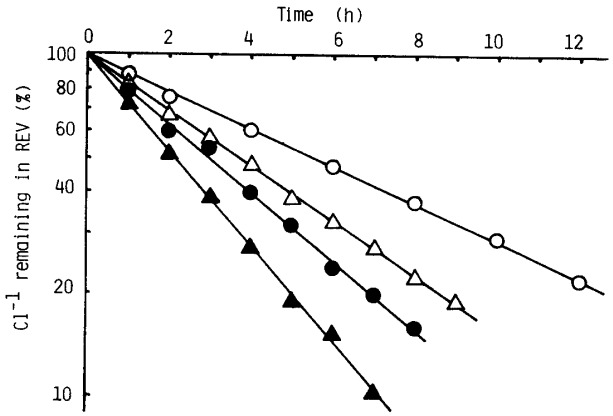

Fig. 6. The Effect of BSA on Chloride Leakage from REV

BSA concentration: ○, 0; △, 5; ●, 20; ▲, 60 mg/ml. Temperature: 37 C. Liposomal lipid: 2.6 μmol/ml.

concentration and followed first-order kinetics up to approximately 80—90% leakage, in contrast to the case with plasma (Fig. 6). However, the effectiveness of BSA was much less than that of plasma, and in contrast with plasma no tendency to saturate was found within the concentration range of BSA examined (Fig. 4). Assuming that human serum albumin (HSA) is as effective in the acceleration as BSA and that plasma contains 40 mg of HSA per ml,[19] the effect of plasma was estimated to be approximately 50 times larger than that of a corresponding amount of HSA. This is consistent with the prior observation that albumin does not affect the liposome permeability as significantly as HDL in plasma.[2-4] The activation energy of the leakage rate accelerated by BSA was comparable to that of the spontaneous rate (Fig. 5).

These results strongly suggest that a different protein–lipid interaction mechanism may operate in human plasma for the destabilization of the lipid bilayer. Several mechanisms can be hypothesized that might account for the difference; BSA molecules may form a permanent access, possibly "water channels," across the bilayer after encountering each other, permitting easier movement of chloride toward the outside as compared with the intact membrane. Meanwhile, the bursting leakage induced by plasma, characterized by a higher activation energy, may be due to disturbance of the bilayer amounting nearly to "physical rupture" of vesicles in the very early stage. The amount of proteins (probably HDL) in plasma participating in this function is considered to be very small, ranging from one-tenth to one-hundredth of the amount of albumin.[19] Although the mechanisms of the bursting leakage caused by such small amounts of proteins and the subsequent restoration of the barrier function are not understood in detail, the incorporation of a larger molecular volume of functional proteins into liposomes might cause considerable disturbance of the bilayer upon the initial interaction between them. After the rearrangement of membrane components, the proteins arising from the plasma may also form "water channels" through the bilayer as in the case of albumin. However, the magnitude of the contribution should be negligible because of their very small content, resulting in the apparent restoration of the barrier function.

References

1) J. Senior and G. Gregoriadis, *Life Sciences*, **30**, 2123 (1982).
2) J. Zborowski, F. Roerdink and G. Scherphof, *Biochim. Biophys. Acta*, **497**, 183 (1977).
3) G. Scherphof, F. Roerdink, M. Waite and J. Parks, *Biochim. Biophys. Acta*, **542**, 296 (1978).
4) J. Damen, J. Dijkstra, J. Regts and G. Scherphof, *Biochim. Biophys. Acta*, **620**, 90 (1980).
5) J. Damen, J. Regts and G. Scherphof, *Biochim. Biophys. Acta*, **665**, 538 (1981).
6) J. Damen, J. Regts and G. Scherphof, *Biochim. Biophys. Acta*, **712**, 444 (1982).
7) C. Kirby and G. Gregoriadis, *Biochem. J.*, **199**, 251 (1981).
8) C. A. Hunt, *Biochim. Biophys. Acta*, **719**, 450 (1982).

9) F. Szoka and D. Papajadjopoulos, *Proc. Natl. Acad. Sci. U.S.A.*, **75**, 4194 (1978).
10) D. Papajadjopoulos and J. C. Watkins, *Biochim. Biophys. Acta*, **135**, 639 (1967).
11) A. D. Bangham, *Annu. Rev. Biochem.*, **41**, 753 (1972).
12) R. D. Kornberg, M. C. MacNamee and H. M. McConnell, *Proc. Natl. Acad. Sci. U.S.A.*, **69**, 1508 (1972).
13) P. Nicholls and N. Miller, *Biochim. Biophys. Acta*, **356**, 184 (1974).
14) Y. Toyoshima and T. E. Thompson, *Biochemistry*, **14**, 1525 (1975).
15) K. Tsukada, R. Okada, M. Hisaoka and T. Morioka, *Chem. Pharm. Bull.*, **30**, 3679 (1982).
16) D. Hoekstra and G. Scherphof, *Biochim. Biophys. Acta*, **556**, 196 (1979).
17) G. Scherphof, H. Morselt, J. Regts and J. C. Wilschut, *Biochim. Biophys. Acta*, **556**, 196 (1979).
18) V. Z. Neitchev and F. A. Bideaud, *Int. J. Biochem.*, **13**, 113 (1981).
19) M. Kitamura, "Seikagaku Data Book I," Tokyo Kagakudojin, Tokyo, 1979, pp. 1546—1547.