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Liposomal Membranes. XVIII. Interaction of Spermicidal Agents with Liposomal Membranes

JUNZO SUNAMOTO,*[,a] KIYOSHI IWAMOTO,[a] HIROAKI IKEDA,[a] and KAZUMARO FURUSE[b]

Department of Industrial Chemistry, Faculty of Engineering, Nagasaki University,[a] Nagasaki 852, Japan and Research and Development Division, Eisai Co., Ltd.,[b] Koishikawa 4-chome, Bunkyo-ku, Tokyo 112, Japan

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The release of a water soluble material, carboxyfluorescein (CF), from the interior of liposomes induced by several spermicidal agents, nonionic surfactants (TS-88, INP-90, and NOP-90), has been investigated. The efficiency of the agents for CF-release was significantly correlated with that for the inhibition of fertilizing ability of sea urchin sperm ($p<0.01$). On the other hand, it was also found that these nonionic surfactants promote the aggregation of liposomes at a concentration just below the cmc. The efficiency and the minimum concentration able to induce both CF-release and the aggregation of liposomes were closely related with not only the hydrophobicity but also the geometry of the hydrophobic moiety of the surfactant.

Keywords——liposome; sperm cell membrane model; spermicidal surfactant; menfegol (TS-88); nonoxynol-9 (INP-90); octoxynol-9 (NOP-90)

Much work have been done on the biological action of surfactants on cell membranes, dealing with spermicidal activity,[1-9] hemolysis,[2] bactericidal activity,[2] and so forth. The biological action of surfactants on the cell membranes is affected by both the concentration and chemical structure of surfactants. In this work, of these various actions of surfactants on cell membranes, the interaction of several spermicidal surfactants with liposomes has been studied to clarify the correlation between their biological activity and chemical structure. There are several stages between the initial reaction with the change in membrane permeability and the final lysis of cell membranes. However, the detailed mechanism of interaction of spermicidal agents with spermatozoa is not fully understood yet, especially at the molecular level.

Koefoed-Johnsen and Mann have already stated that ionic surfactants, hexadecyltrimethylammonium bromide (CTAB) and sodium dodecyl sulfate (SDS), inhibit the mobility, fructolysis, and respiration of spermatozoa and concomitantly induce a leakage of cytochrome c from the sperm cell.[3] These previous findings encouraged us to investigate the correlation between the structural characteristics of surfactants and their spermicidal activity in conjunction with the change in the permeability of membranes. To this end, egg phosphatidylcholine liposomes, recognized as a convenient artificial cell, were employed as a model of sperm cells. The change in the permeability of liposomes induced by interaction with surfactants was detected by fluorometrically monitoring the leakage of carboxyfluorescein (CF)[10,11] from the interior of liposomes. In this study, the following five surfactants were employed; *p*-(menthanyl)phenyl polyoxyethylenyl (8.8) ether (TS-88), *p*-(1,3,5-trimethylhexyl)phenyl polyoxyethylenyl (9) ether (INP-90), *p*-(1,1,3,3-tetramethylbutyl)phenyl polyoxyethylenyl (9) ether (NOP-90), SDS, and CTAB (Fig. 1). The first three surfactants are referred to by their trivial names of menfegol, nonoxynol-9, and octoxynol-9, respectively, and they have been demonstrated to show high spermicidal activity, and low toxicity and ir-

ritancy in the vagina.[1,2,6-9] These nonionic surfactants have the same polyethylene oxide moiety as the hydrophilic head, while the structure of the hydrophobic moiety is different in each of them.

On the other hand, we also found that these spermicidal surfactants promote the aggregation of multilamellar liposomes at a concentration just below the critical micellar concentration (cmc). In this article, hence, the mechanism and efficiency of the induced aggregation will also be briefly discussed.

Experimental

Materials——Egg phosphatidylcholine (egg PC) was isolated and purified from fresh egg yolk according to the method described previously.[12,13] Hexadecyltrimethylammonium bromide (CTAB) and sodium dodecyl sulfate (SDS) (Wako Pure Chemical Industries, Ltd., Osaka) were recrystallized from ether–methanol (5:2, by vol.) and ether–ethanol (5:2, by vol.), respectively. NOP-90 and INP-90 were gifts from Nihon Emulsion Co., Ltd., Tokyo and Sanyo Kasei Kogyo Co., Ltd., Kyoto, respectively. TS-88 was synthesized from ethylene oxide and *p*-menthanylphenol derived from turpentine oil. All the nonionic surfactants employed in this work were the same as those used in the previous work.[14,15] The isomer ratios of the *ortho* and *para* substituents of INP-90 and NOP-90 are shown in Fig. 1. Cmc of NOP-90, INP-90, and TS-88 were found to be 0.4, 0.6, and 0.7 mM, respectively, in an aqueous solution containing 5% (w/v) glucose (Glucose Injection, JP X) at 25.0 °C.[15] Carboxyfluorescein (CF) was purchased from Eastman Kodak, Rochester, N.Y. Other reagents were commercially available as analytical grade and used without further purification.

Preparation of Liposomes——Small single-walled liposomes which contain 200 mM CF in the interior were prepared by essentially the same method as described before.[16] Gel-filtration of liposome suspensions was carried out on a Sepharose 4B column (ϕ1.8 i.d. × 41 cm) pre-equilibrated in 20 mM Tris-HCl buffer (pH 8.6) containing 200 mM sodium chloride. The concentration of liposomes was determined as inorganic phosphate according to Allen's procedure.[17]

Monitoring the Release of CF from Liposomes——Fluorescence measurements were run on a Hitachi 650-10S fluorospectrophotometer equipped with a thermoregulated cell compartment. CF emits at 520 nm on excitation at 470 nm. A suspension of small single-walled liposomes containing 200 mM CF in the interior hardly fluoresces, but fluoresces strongly upon liberation from the concentration quenching when CF is released from the interior to the bulk aqueous phase. Hence, the reduced barrier function of liposomal bilayers can be observed quantitatively by monitoring an increase in the fluorescence intensity of CF at 520 nm. The total amount of CF encapsulated in liposomes was determined by completely destroying the liposomes by adding 30 μl of 10% (v/v) aqueous Triton X-100 solution to 1.0 ml of the liposome suspension.[10,11]

All the runs were initiated by adding an aqueous buffered (pH 8.6) solution of a surfactant to a liposome suspension after preincubation for 15 min at 37.0 °C (physiological temperature). The amount of released CF was calculated by means of the following equation: $\%CF\ release = (I_t - I_0)/(I_\infty - I_0)$

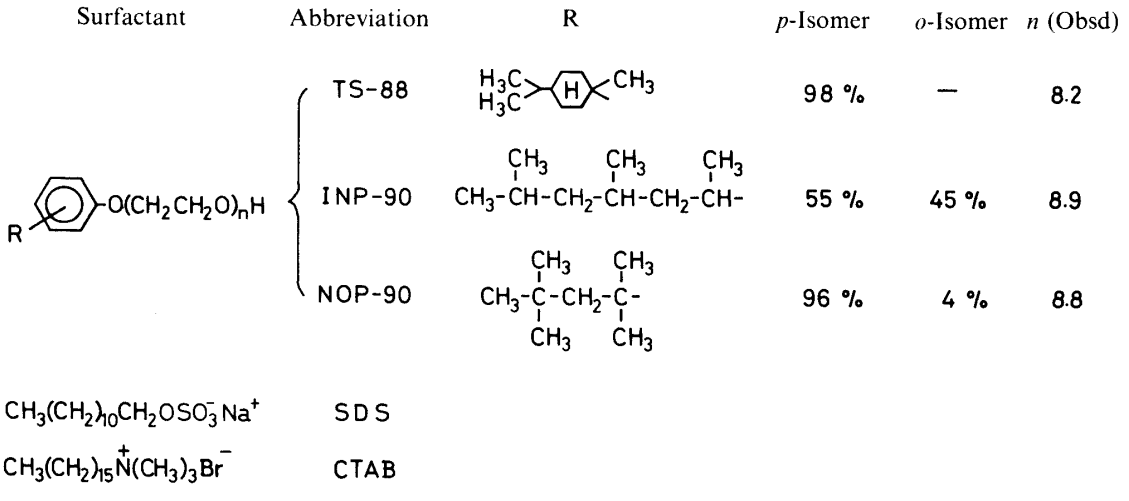

Fig. 1. Structures of Surfactants Employed in This Work

where I_0 is the fluorescence intensity of CF-loaded liposome suspension at 520 nm in the absence of any surfactant, while I_∞ is the fluorescence intensity at 520 nm after destroying the liposomes by Triton X-100. I_t stands for the fluorescence intensity at 520 nm at time t after adding a surfactant solution to a liposome suspension.

Aggregation of Liposomes——Aggregation of multilamellar liposomes induced by nonionic surfactants was followed by monitoring the turbidity at 600 nm on a Hitachi 200 spectrophotometer.[18,19] Aggregation started when an aqueous buffered (pH 8.6) solution of surfactant was injected to a liposome suspension after preincubation at 37.0 °C.

Determination of cmc——The cmc's of surfactants employed were determined from a plot of surface tension obtained by the drop-weight method (JIS K 3362-1970)[14] as a function of the surfactant concentration. Surfactants were dissolved in 5% (w/v) aqueous glucose solution and measurements were carried out at 37.0±0.5 °C.

Results and Discussion

It was found that all the surfactants employed in this work induce the release of a water soluble material, CF, from the interior of liposomes. Figure 2 shows a typical example of the detergent-induced CF release from liposomes as a function of time. Upon the addition of surfactant to a liposome suspension, rapid release of CF was observed at the initial stage followed by a slow release which finally leveled off. No spontaneous release of CF was observed during the preincubation. Most probably, the fast CF-release caused by adding a surfactant solution at the initial stage is produced by perturbation of the bilayer structure upon adsorption of the surfactant on the membrane surface.[20] Subsequent slow release would be brought about by a decreased barrier function of membranes with increased fluidity upon permeation of the surfactants into and their diffusion in bilayer membranes.[20,21]

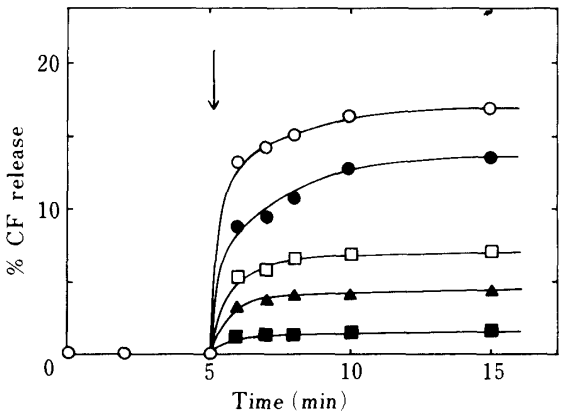

Fig. 2. Release of Carboxyfluorescein (CF) Induced by Various Surfactants from Small Single-Walled Liposomes of Egg PC in 20 mM Tris-HCl Buffer (pH 8.6) as a Function of Time at 37.0 °C

An arrow shows the point of injection of surfactant solution. [egg PC] = 1.3×10^{-4} M; [surfactant] = 5.0×10^{-5} M; (—○—), TS-88; (—●—), INP-90; (—□—), NOP-90; (—▲—), CTAB; (—■—), SDS.

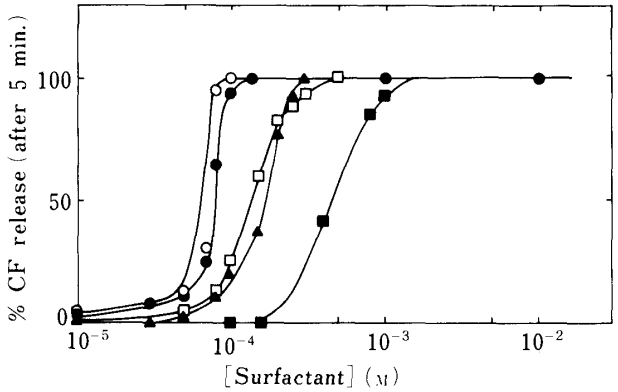

Fig. 3. Induced Release of Carboxyfluorescein (CF) (% Release at 5 min after Adding Surfactant Solution) from Single-Walled Liposomes of Egg PC as a Function of Surfactant Concentration in Tris-HCl Buffer (pH 8.6) at 37.0 °C

[egg PC] = 1.3×10^{-4} M; (—○—), TS-88; (—●—), INP-90; (—□—), NOP-90; (—▲—), CTAB; (—■—), SDS.

The extent of the induced CF-release was due to the concentration of the surfactant added (Fig. 3). Under the conditions employed, the surfactant induced release of CF was observed even at a relatively low surfactant concentration below the cmc (cmc values are 0.71 mM for TS-88, 0.62 for INP-90, 0.52 for NOP-90, 7.10 for SDS, and 2.88 for CTAB at 37.0 °C). This means that the release of CF is brought about by permeation of monodispersed surfactant molecules into liposomal bilayers, not by solubilization of liposomes by surfactant micelles. This is also supported by the electron microscopic observation that a vesicular structure was maintained even in the presence of surfactants under the conditions used. Figures 2 and 3 indicate that the sequence in the efficiency of surfactants for CF-release is TS-88 > INP-90 > NOP-90 ≧ CTAB > SDS. This is not correlated with the cmc of the surfactants. In the case of the present three spermicidal agents, the efficiency seems to depend on the geometry of the hydrophobic moiety of the surfactant. A surfactant carrying a bulkier hydrophobic moiety causes larger release of CF from the liposomes. It is reasonable, therefore, that the *o*-isomer seems more potent to purturb the liposomal bilayers than the *p*-isomer when it has penetrated into the membranes (Figs. 1 and 3). The correlation between the isomer ratio and the efficiency for induced CF-release with INP-90 and NOP-90 may again reflect the bulkiness of the hydrophobic part of the surfactant (including the phenyl ring in the cases of INP-90 and NOP-90).

Recently, one of the present authors (K. F.) has shown that the sequence of inhibitory effect of surfactants on the fertilizing ability of sea urchin sperm is TS-88 > INP-90 ≧ NOP-90 > CTAB > SDS.[15] This is identical with that of the efficiency for promotion of CF release from liposomes. Table I shows that there exists a good correlation between the effective concentration of the surfactant for inhibition of fertilizing ability of sea urchin sperm (C_{f_0}) and that for induced 100% CF-release from liposomes ($C_{r_{100}}$). From the data in Table I, the following linear relationship could be established: $\log C_{f_0} = 0.826 \log C_{r_{100}} - 2.23$, with $r = 0.969$, $n = 5$, and $p < 0.01$. These results suggest that liposomes are a useful model for sperm cells and can be used to evaluate the ability of surfactants to alter the permeability of sperm cell membranes, which is an important factor determining the spermicidal activity.

Addition of a nonionic surfactant to a suspension of egg PC multilamellar liposomes induced the aggregation of liposomes, which was followed spectrophotometrically by monitoring an increase in the turbidity of the liposome suspension. Figure 4 shows a typical example of the surfactant-induced aggregation of multilamellar liposomes as a function of time. After a relatively rapid increase in the turbidity upon the injection of nonionic surfactant, the increase in the turbidity gradually leveled off. When an egg PC multilamellar liposome suspension was incubated in the absence of surfactants, no increase in the turbidity was observed at all over a period of several hours. The efficiency of these surfactants for

Table I. Effective Concentrations of Surfactants for Induced CF-Release from Liposomes and for 100% Inhibition of Fertilizing Ability of Sea Urchin Sperm

Surfactants	$C_{r_{100}}$ [a] (mM)	C_{f_0} [b] (μM)
TS-88	0.10	2.88
INP-90	0.15	5.50
NOP-90	0.30	5.37
CTAB	0.50	10.47
SDS	1.50	31.62

a) Effective molar concentration that induces 100% CF-release from liposomes.
b) Effective molar concentration for the 100% inhibition of fertilizing ability of sea urchin sperm.[15]

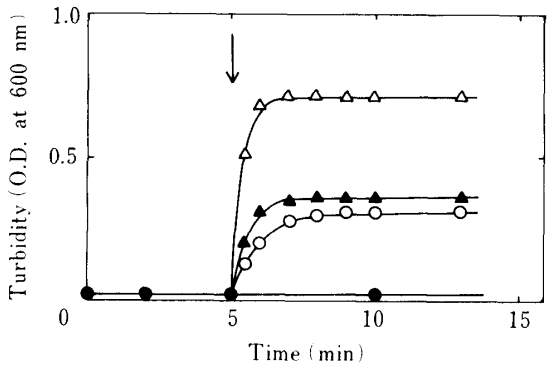

Fig. 4. A Typical Example of the Turbidity Change upon Aggregation of Egg PC Multilamellar Liposomes ([Egg PC] = 1.3×10^{-4} M) Induced by TS-88 (—●—, 3.0×10^{-4} M; —○—, 6.0×10^{-4} M; —△—, 7.0×10^{-4} M; —▲—, 1.5×10^{-3} M) in 10 mM Tris-HCl Buffer (pH 8.6) Containing 0.10 M Sodium Chloride at 37.0 °C

A vertical arrow indicates the point at which an aqueous TS-88 solution was injected.

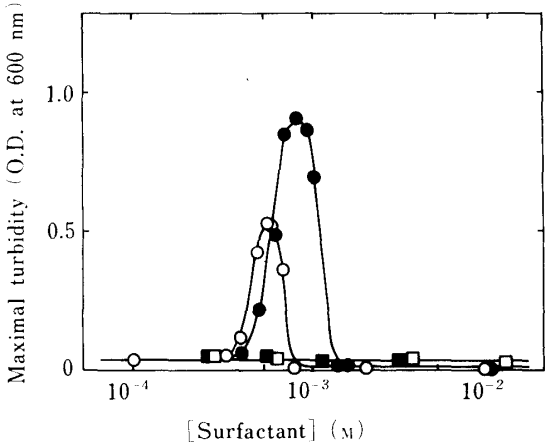

Fig. 5. Relationship between the Maximal Turbidity and the Concentration of Surfactant in the Induced Aggregation of Multilamellar Liposomes ([Egg PC] = 1.3×10^{-4} M) in 10 mM Tris-HCl Buffer (pH 8.6) Containing 0.10 M Sodium Chloride at 37.0 °C

—●—, INP-90; —○—, TS-88; —■—, CTAB; —□—, SDS.

inducing aggregation of multilamellar liposomes was also related to the concentration of surfactant (Fig. 5). The addition of INP-90 up to 4×10^{-4} M did not induce any aggregation of liposomes. Further addition up to 8×10^{-4} M caused a rapid increase in the maximal turbidity (Fig. 5). Addition of INP-90 above 8×10^{-4} M led to a recovery of the clarity of the mixed suspension, which indicates that the multilamellar liposomes are gradually destructed by INP-90 micelles. Finally, the turbidity completely disappeared on adding the surfactant at a concentration above 1.3×10^{-3} M. These results indicate that the multilamellar liposomes can be completely solubilized by INP-90 micelles. Similar phenomena were observed for TS-88 though the extent of maximal turbidity and the minimum concentration required to induce the aggregation were different (Fig. 5). TS-88 induces the aggregation of multilamellar liposomes at a lower concentration than INP-90. This difference between TS-88 and INP-90 was consistent with that in the efficiency of induction of CF-release from liposomes by the two surfactants. The maximal turbidity induced by TS-88 was rather smaller than that induced by INP-90; this difference is apparently not related to their cmc values. This may arise because TS-88 more easily forms mixed micelles with egg PC even at a concentration below the cmc and consequently solubilizes more liposomes and/or liposome aggregates even at relatively low concentration. Again the geometrical difference as well as the difference in hydrophobicity between the two surfactants must be considered as important factors. On the other hand, ionic surfactants, CTAB and SDS, did not induce any aggregation of multilamellar liposomes under the same conditions (Fig. 5). This may be due to the electrostatic repulsion between the charged surfaces of liposomes produced by the ionic detergents after permeation into the egg PC liposomal bilayers.

Considering that liposomes composed of neutral glycerol–glycolipids spontaneously aggregate[22] and polyethylene glycol induces cell–cell aggregation,[23] the present aggregation of multilamellar liposomes may be interpreted in terms of the reduced repulsion between liposomes caused by covering the liposome surface with the hydrophilic polyethylene oxide moieties of the nonionic surfactants permeated into the liposomal bilayers and/or by an interliposomal interaction *via* polyethylene oxide moieties of surfactant molecules at the liposome surface.

In any event, the results obtained in this work indicate that liposomes can be used to estimate the spermicidal activity of surfactants as well as to design potent inducers to promote cell–cell aggregation and fusion.

References

1) S. Iwahara, C. Ishizeki, and K. Furuse, *Med. Biol.* (Tokyo), **73**, 98 (1966).
2) K. Furuse, *Yukagaku*, **18**, 114 (1969).
3) H. H. Koefoed-Johnsen and T. Mann, *Biochem. J.*, **57**, 406 (1954).
4) P. E. Lindahl and K. Wedin, *Nature* (London), **184**, 1289 (1959).
5) C. Harvey and P. E. Stuckey, *J. Reprod. Fertil.*, **3**, 124 (1962).
6) D. A. Berberian, W. H. Gorman, H. P. Drobeck, F. Coulston, and R. G. Slighter, Jr., *Toxicol. Appl. Pharmacol.*, **7**, 206 (1965).
7) D. A. Berberian, W. G. Gorman, H. P. Drobeck, F. Coulston, and R. G. Slighter, Jr., *Toxicol. Appl. Pharmacol.*, **7**, 215 (1965).
8) S. Iwahara, C. Ishizeki, and K. Furuse, *Jpn. J. Public Health*, **12**, 123 (1965).
9) M. Chvapil, J. B. Ulreich, K. O'Dea, K. Betts, and W. Droegemueller, *Fertil. Steril.*, **33**, 521 (1980).
10) J. N. Weinstein, S. Yoshikami, P. Henkart, R. Blumenthal, and W. A. Hagins, *Science*, **195**, 489 (1977).
11) F. C. Szoka, Jr., K. Jacobson, and D. Papahadjopoulos, *Biochim. Biphys. Acta*, **551**, 295 (1979).
12) W. S. Singleton, M. S. Gray, M. L. Brown, and J. L. White, *J. Am. Oil Chem. Soc.*, **42**, 53 (1965).
13) J. Sunamoto, H. Kondo, and A. Yoshimatsu, *Biochim. Biophys. Acta*, **510**, 52 (1978).
14) K. Furuse, C. Ishizeki, and S. Iwahara, *J. Pharm. Dyn.*, **6**, 359 (1983).
15) E. Nakano, M. Hino, and K. Furuse, *Gamete Res.*, "submitted."
16) K. Iwamoto and J. Sunamoto, *Bull. Chem. Soc. Jpn.*, **54**, 399 (1981).
17) R. J. L. Allen, *Biochem. J.*, **34**, 857 (1940).
18) J. Sunamoto, K. Iwamoto, and H. Kondo, *Biochem. Biophys. Res. Commun.*, **94**, 1367 (1980).
19) J. Sunamoto, K. Iwamoto, H. Kondo, and S. Shinkai, *J. Biochem.* (Tokyo), **88**, 1219 (1980).
20) K. Iwamoto and J. Sunamoto, *J. Biochem.* (Tokyo), **91**, 975 (1982).
21) V. Viti and M. Minetti, *Chem. Phys. Lipids*, **28**, 215 (1981).
22) K. Iwamoto, J. Sunamoto, K. Inoue, T. Endo, and S. Nojima, *Biochim. Biophys. Acta*, **691**, 44 (1982).
23) S. Sasakawa and K. Honda, *Membrane*, **6**, 310 (1981).