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Detergent and Phospholipid Mixed Micelles as Proliposomes for an Intravenous Delivery of Water-Insoluble Drugs

Kyonghee Son

Research & Development Center, Daewoong Pharmaceutical Co., Ltd.
223-23 SangDaewon-Dong, Joongwon-Gu, Sungnam, Kyunggi-Do 462-120, Korea
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Abstract

A novel drug delivery system, detergent-phospholipid mixed micelles as proliposomes, for water-insoluble compounds was developed by investigating (i) spontaneous formation of small unilamellar vesicles (SUV) from bile salt-egg phosphatidylcholine mixed micelles, (ii) the molecular mechanism of micelle-to-vesicle transition in aqueous mixtures of detergent-phospholipid, (iii) preparation and screening of a suitable liposomal formulation for a lipophilic drug: solubilization of the drug within the lipid bilayer, evaluation of the solubility limit, and characterization of the resulting product with respect to the physical properties and stability of the drug in the system, and (iv) testing antitumor activity *in vitro*. The results showed that the new carrier had a strong possibility to be a biocompatible universal formulation for water-insoluble drugs.

1. INTRODUCTION

The intravenous administration of insoluble compounds in a challenge for pharmaceutical scientists. The preferred vehicle for i.v. administration is always water. However, a significant number of compounds exhibit inadequate solubility in water. Classical liposomal preparations are currently under investigation for the i.v. delivery of some insoluble drug compounds (Ostro, 1983; Su *et al.*, 1981). However, both *in vivo* and *in vitro* stabilities of liposomal products still need to be improved and a large batch production of a reproducible liposomal product has not been achieved. We have investigated a novel approach to the i.v. delivery of a water-insoluble compound. The new carrier system is composed of mixed micelles of two amphiphiles: a water-soluble amphiphile (e.g., detergent) and a water-insoluble amphiphile (e.g., phosphatidylcholine). This system is capable of solubilizing hydrophobic compounds, for instance, cholesterol (Mazer and Carey, 1983). Bile salts are biological detergents that have amphiphilic properties and form membrane-toxic simple micelles in aqueous media above the critical micelle concentration (CMC). In the presence of phospholipid, bile salts form thermodynamically stabile mixed micelles that are less toxic (Carey and Small, 1970; Teelemann *et al.*, 1984). Unilamellar phospholipid vesicles of 30 to 200 nm diameter are widely used as models for biological membranes, drug delivery systems and substrates for a variety of enzymes and proteins (Frendler, 1983). It was previously shown by quasi-elastic light scattering (QELS) that bile salt and egg phosphatidylcholine (PC) mixed micelles were spontaneously transformed into vesicles upon aqueous dilution (Schurtenberger *et al.*, 1985; Donovan *et al.*, 1987) or equilibrium dialysis (Schurtenberger, 1985). Vesicles are also formed from mixed detergent-phospholipid micelles after the removal of detergent by gel filtration or treatment with detergent adsorbers (Romer-luthy *et al.*, 1980). Octylglucoside-egg PC mixed micelles

produce larger vesicles (Mimms *et al.*, 1981) than bile salt-egg PC mixed micelles. There is some evidence of intermediate structures during the vesicle formation in bile salt/egg PC mixtures. Hjelm *et al.* (1988) demonstrated that the shape of the mixed micellar particles changed from roughly spherical to rod-like during the micelle-to-vesicle transition using small-angle neutron scattering analysis. Walter *et al.* (1990) found, using cryotransmission electron microscopy, that, as cholate was added, open vesicles and long rod-shaped micelles coexisted until all the egg PC was in spheroidal mixed micelles.

Our goal was to investigate the potential of our detergent-phospholipid mixed micelle model, as a novel and stabile delivery system for water-insoluble drugs. Teniposide (VM 26, chemically 4-demethyl-epipodo-phyllotoxin-β-D-thenylidene glucoside), water-insoluble, was chosen as a model drug. It is a natural product found in the root of the American mandrake (*Podophyllum peltatum*) and has been shown to be active against a variety of solid tumors, leukemias, lymphomas (Hodgkin's disease and non-Hodgkin's lymphoma) and neuroblastoma (O'Dwyer *et al.*, 1984). The molecular interactions, such as electrostatic and hydrophobic interactions between detergent and phospholipid molecules responsible for vesicle formation from micelles, were examined. These results were used to formulate a lipophilic model drug, teniposide. The bile salt-egg PC system was initially chosen because of its well-defined phase diagram.

2. EXPERIMENTAL METHODS

2-1. Materials

Sodium glycocholate (GC), sodium taurocholate (TC), sodium cholate (C), sodium chenodeoxycholate (CDC), sodium glycochenodeoxycholate (GCDC), sodium taurochenodeoxycholate (TCDC), sodium deoxycholate (DOC), sodium taurodeoxycholate (TDOC), sodium glycodeoxycholate (GDOC), sodium deoxycholate (DOC), 3-[(3-cholanidopropyl)-dimethylammonio]-1-propanesulfonate(CHAPS), egg yolk and soy bean phosphatidylcholine (PC), egg yolk phosphatidylethanolamine (PE), phosphatidic acid (PA), and cholesterol were purchased from Sigma Chemical Co. (St. Louis, MO) and used without further purification. N-Octylglucoside was purchased from Boehringer Mannheim (Mannheim, W. Germany). Dodecyltrimethylammonium bromide (DTAB) was purchased from Aldrich. Trisma base and Tris-HCl (Sigma), N-2-hydroxy-ethylpiperazine-N'-2-ethanesulfonic-acid (Hepes) (Sigma), 3-[(N-morpholine)]-propane-sulfonic acid (Mops) (Sigma), cyclohexylamino-ethanesulfonic acid (CHES) (Sigma), imidazole, sodium citrate, citric acid, sodium mono- and di-basic phosphate, and sodium chloride (Fisher) were all analytical grade. Teniposide was a gift and used as received from Bristol-Myers Laboratories (Syracuse, NY). Human serum was purchased from Hazelton Research Products (Lenexa, Kansas). Human serum albumin and immunoglobulin G were purchased from Sigma. Human lipoproteins were purified from human serum by the phosphotungstate precipitation method (Burstein *et al.*, 1970).

2-2. Preparation of Solutions

Aqueous detergent-phospholipid mixed micellar solutions were prepared by the method of coprecipitation (Small *et al.*, 1969) unless otherwise noted. Detergents and egg PE were dissolved in methanol and egg PA in chloroform/methanol (1:1, v/v) as stock solutions while egg yolk and soybean PC were dissolved in ethanol. The appropriate concentrations of each stock solution were mixed, thoroughly dried under a stream of purified nitrogen and then dried *in vacuo* for 2-3 days, until a constant dry weight was obtained. The appropriate aqueous solvents were added to the dried film to give the desired total phospholipid plus detergent concentration. All reconstituted micellar stock

solutions were flushed with nitrogen, sealed, and equilibrated for 2 days at the specified temperature. The solutions for QELS studies were prepared by aqueous dilution from the micellar stock solutions with diluents which were identical with the reconstitution solvents. Each solution was likewise flushed with nitrogen, sealed, and left for 2 days at the specified temperature to reach equilibrium.

2-3. Quasi-Elastic Light Scattering (QELS) Measurements

These were performed utilizing a Nicomp Model 270 Submicron particle sizer (Pacific Scientific, Menlo Park, CA) equipped with a 5 mW Helium-Neon Laser at an exciting wavelength of 632.8 nm and with a 64-channel autocorrelation function, a temperature-controlled scattering cell holder and an ADM 11 video display terminal computer (Lear Siegler Inc., Anaheim, CA) for analyzing the fluctuations in scattered light intensity generated by the diffusion of particles in solution. The mean hydrodynamic particle diameter, $\bar{d}_h$, was obtained from the Stokes-Einstein relation using the measured diffusion coefficient obtained from each fit. The experimental results are the average of three $\bar{d}_h$ values obtained from analysis of autocorrelation functions accumulated for 30 min. The following instrument settings were used: temperature, 23℃; viscosity, 0.9325 cp; refractive index, 1.333; scattering angle, 90°; and size range, 15-200 nm.

2-4. Trapped Volume and Trapping Efficiencies

Vesicles were prepared in the presence of cytosine arabinoside (5 mg/m*l*), water-soluble drug, in the diluent media. Untrapped drug was removed by extensive dialysis against drug-free buffer (0.02 M Tris, pH 7.5 containing 0.15 M NaCl and 0.001 M NaN_3) at room temperature using Spectrapor 2 dialysis membrane tubing with a molecular weight cutoff of 12,000 (Spectrum Medical Industries, Inc.). The concentrations of cytosine arabinoside trapped within vesicles was determined by high performance liquid chromatography (HPLC) after breaking down the liposomal membrane with methanol. Reverse-phase HPLC was performed, using a system consisting of a Waters 600 pump fitted with a U6K manual injector, a RCM-100 module, a 490-programmable multiwavelength detector and 745 data module (Millipore). A Novapak C_{18} Radial-Pak column (particle size 4 μm, 10 cm length×8 mm internal diameter) was used. The mobile phase was degassed with high purity helium (Union Carbide, Linde Division). The flow rate was 1.0 m*l*/min and detection was made at 254 nm and 0.500 AUFS. Trapped volumes were calculated based on the diameter of vesicles, assuming the bilayer thickness of 4 nm and the area per phospholipid molecule of 0.7 nm^2 (Enoch and Strittmatter, 1979; Cullis *et al*., 1987). Trapping efficiencies were calculated from the ratio of the drug concentrations before and after removal of untrapped drug.

2-5. Transmission Electron Microscopy (TEM)

Diameters of mixed micelles and mixed vesicles were determined by TEM following staining. A small drop of the sample in Tris buffer (pH 7.5) was placed onto a carbon-coated copper grid. Excess sample was drained off with filter paper, leaving a thin film of the samples on the grid. One drop of 1% phosphotungstic acid buffer was added to the grid and allowed to stand for 1 min. Excess solution was drained off with filter paper and allowed to air dry for 2-3 min. Samples were viewed on a Jeol Jem 100S transmission electron microscope and photographed at 160,000× magnification. At least 700 particles were measured on several electron micrographs covering different areas on a grid.

2-6. Freeze-Fracture Transmission Electron Microscopy

Vesicles, prepared by diluting the stock micellar solution (egg PC/TC=1.0, total egg PC plus taurocholate concentration=50 mg/m*l*) with 0.02 M Tris (pH 7.5) containing 0.15

M NaCl, 0.001 M NaN_3 and 25% (v/v) glycerol in the absence and presence of teniposide, were sandwiched between two copper planchettes followed by freezing, fracturing, and replicating according to Mayer *et al.* (1986) and Kaler *et al.* (1989). The replicas were viewed on a Jeol Jem 100S transmission electron microscope and photographed at 40,000× magnification.

2-7. Preparation of Teniposide-Containing Micelles and Liposomes

Aqueous bile salt-egg PC-teniposide mixed micellar solutions were prepared by coprecipitation. Bile salt and phospholipid were dissolved in ethanol and teniposide in a chloroform/methanol mixture (2:1) as stock solutions. Stock solutions were mixed and solvent evaporated under a stream of purified nitrogen and then dried under vacuum for 2-3 days to constant dry weight. The appropriate aqueous solvents were added to give the desired total lipid concentration. The solutions were flushed with nitrogen, sealed and equilibrated for one day at 10℃. To remove unsolubilized teniposide, the solutions were centrifuged at 12,000g for 5 min. Liposomal solutions were prepared by diluting the stock micellar solutions with the buffer.

2-8. Measurement of Teniposide Solubilized in Mixed Micelles and Liposomes

The concentrations of teniposide encapsulated within micelles and vesicles were determined by reverse-phase high performance liquid chromatography (HPLC). The mobile phase was methanol and water (70:30, v/v), degassed with high purity helium (Union Carbide, Linde Division). The flow rate was 1.0 m*l*/min. and the detection was made at 240 nm and 1.000 AUFS.

2-9. Stability Studies

Short-term stability studies of teniposide in mixed micelles and in liposomes were carried out under different conditions Teniposide-containing mixed micellar and/or liposomal solutions were stored at different temperatures (eg., 10°, 23°, and 45℃) or in the freeze-dried or frozen state. Samples were taken at the predetermined time intervals and centrifuged at 12,000g for 5 min. to remove the precipitated teniposide. The solutions were frozen at −20℃ in dry ice/isopropanol and stored at −20℃ or lyophilized for two days at −50℃ under reduced pressure (10 microns Hg) (Beckman) and stored at 10 ℃. Samples were thawed or reconstituted with water followed by centrifugation. Teniposide remaining in micellar and liposomal solutions was determined by reverse-phase HPLC The mobile phase was methanol and water (70:30, v/v) which were degassed with high purity helium (Union Carbide, Linde Division). The flow rate was 1.0 m*l*/min. and the detection was made at 240 nm.

The mean hydrodynamic diameter ($\bar{d}_h$) of mixed micelles (PC/TC=0.8, total lipid conc. =10 mg/m*l*) and liposomes (PC/TC=0.8, total lipid conc.=1.67 mg/m*l*) stored at 10℃ or frozen or lyophilized were measured.

2-10. An *in vitro* Bioassay

S180 murine sarcoma cells (CCL 8, passage 68) (the American Type Culture Collection, Rockville, MD) were maintained *in vitro* in Eagle's minimal essential medium with non-essential amino acids (Eagle's basal salts, 5% calf serum, 100 U/m*l* of penicillin and 100 μg/m*l* of streptomycin (CMEM-E)) in an atmosphere of 5% air and 95% CO_2. For *in vitro* bioassay of teniposide efficacy, 1×10^6 viable S180 cells (0.9 m*l*) admixed with/without various concentrations of teniposide (0. 1 m*l*) were distributed into the center eight wells of a 24-well cluster plate (Costar, Cambridge, MA) and incubated at 37℃ for 24 hrs. Following incubation, the wells were washed with Dulbecco's phosphate-buffered saline without Ca^{2+} and Mg^{2+} (PBS) and the cells removed and placed in 0.25% trypsin in 1 mM EDTA (Gibco Lab., Grand Island, NY). Numbers of viable cells were determined by counting trypan blue dye-excluding cells in a hemacytometer.

3. RESULTS AND DISCUSSION

3-1. Liposomes Prepared Dynamically between Bile Salt and Phospholipid Molecules

The change in the mean hydrodynamic diameter, $\bar{d}_h$, of the particles in GC-egg PC mixed micellar solution (initial total lipid concentration 50 mg/m*l*, egg PC/GC=0.8) upon dilution is shown in Fig. 1. When the mixed micellar system was diluted around the micellar phase limit (dilution factor of 10), micellar $\bar{d}_h$ values increased strongly from 22 nm to 140 nm. With further dilutions, $\bar{d}_h$ values decreased toward an asymptotic value of approx. 34 nm, consistent with the vesicle sizes obtained by others (Schurtenberger *et al.*, 1985; Donovan *et al.*, 1987). These results demonstrate that bile salt-egg PC mixed micelles are spontaneously transformed into bile salt-egg PC vesicles (Donovan *et al.*, 1987; Son and Alkan, 1989; Mazer and Carey, 1983).

It is of interest that the transition phenomenon was not affected at all by a hydrophilic compound (cytosine arabinoside) present in the diluent buffer (Fig. 1). Furthermore, when the mixed micellar solution was diluted with buffer containing the intermicellar concentration (IMC) of glycocholate (2.1 mg/m*l*, 4.3 mM), the micellar sizes did not change and no transition was observed. This is shown by the lower horizontal line in Fig. 1.

Cytosine arabinoside was chosen as a model drug to calculate the trapping capacity and to compare it with the value reported in the literature. The amount of cytosine arabinoside experimentally entrapped inside vesicles can be determined from the calculated internal volume. The theoretical values of the trapped volume were determined from the diameters of the vesicles that were known from QELS measurements, simply using the relationship between vesicle diameter and internal volume (Enoch and Strittmatter, 1979; Cullis *et al.*, 1987). We assumed that vesicles (dilution factor of 20 and 30) consisted

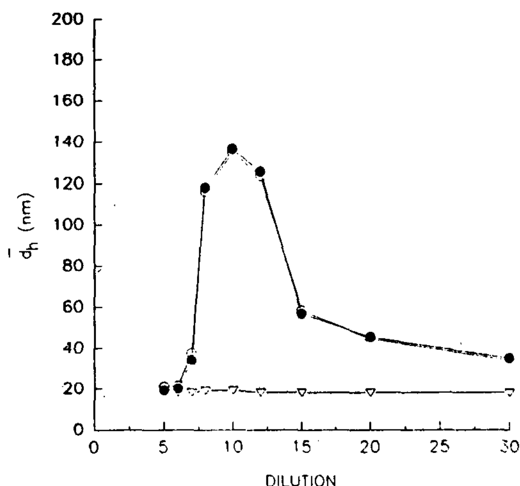


Figure 1—Effect of dilution on mean hydrodynamic diameter, $\bar{d}_h$, of GC-egg PC mixed micellar solution (initial total GC plus egg PC conc.=50 mg/m*l*, egg PC/GC=0.8) at 23° in buffer (0.02 M Tris, pH 7.5, 0.15 M NaCl 0.001 M NaN_3); dilution buffer alone (○), dilution buffer with cytosine arabinoside (5 mg/m*l*) (●) and diluent buffer containing GC (2.1 mg/m*l*, 4.3 mM) (▽). N=3.

of purely phospholipid bilayers since bile salts were diluted far below the critical micellar concentration. The trapped volume and the trapping efficiencies of the vesicles prepared in three dilution media (buffer, 5% and 10% serum containing cytosine arabinoside) at two different lipid concentrations are shown in Table I. Final vesicles (dilution factor of 30) formed in buffer had approximately 10% trapping efficiency which is significantly higher when compared to that obtained by sonication of an aqueous suspension of phospholipids (1-2% efficiency) (Cullis *et al.*, 1987). Interestingly, the vesicles produced in 5% and 10% serum have approximately 2.5% trapping efficiency. This be due to binding and/or interaction of cytosine arabinoside and the phospholipid bilayer with serum components. The trapping efficiency of the vesicles formed in buffer at the dilution factor of 20 was decreased to approximately 1%, indicating that the resides were not completely sealed and some leakage occurred.

Table I—Trapped Volumes and Trapping Efficiencies of Vesicles Formed by Dilution at Two Different Lipid Concentrations[a]

Sample		Lipid concn. (mg/ml)	Trapped-volume (μl/μmol lipid)	Trapping efficiency (%)[b]
dilution medium	dilution factor			
buffer	20	1.40	1.38	7.32± 0.22
	30	0.93	1.08	9.72± 0.28
5% serum	20	1.40	2.64	2.40± 0.10
	30	0.93	2.64	2.51± 0.12
10% serum	20	1.40	3.00	2.46± 0.09
	30	0.93	3.00	2.43± 0.11

[a]The experimental procedure for calculating trapped volume (μl/μmol lipid) and trapping efficiency for cytosine arabinoside were described under Methods.
[b]Numbers represent the results of three experiments (Mean± S.E.)

Table II—The Comparison of the Particle Diameter from Quasi-Elastic Light Scattering (QELS) and Transmission Electron Microscopy (TEM)

Dilution factor	QELS diameter ($\bar{d}_h$, nm)	TEM diameter (nm)
5	22	10-40 (18)[a]
8	99	50-150 (91)
10	140	80-200 (134)

[a]Numbers in parenthesis represent the mean of 700-800 particles.

3-2. Electron Microscopy of GC-Egg PC Solutions

Transmission electron microscopic studies were conducted in order to examine the morphological changes in the particles produced upon dilution during the transition from mixed micelles to mixed vesicles. The electron micrographs of three diluted samples, dilution factor of 5 (mixed micelles, A), 8 (mixed micelles, B) and 10 (mixed vesicles, C) with corresponding $\bar{d}_h$ values measured by QELS of 22, 99 and 140 nm, respectively are shown in Fig. 2.

As shown in Table II, there is close correlation between the mean hydrodynamic diameter, $\bar{d}_h$ measured by QELS (Fig. 1) and the TEM diameter (Fig. 2). We have also found that there were no morphological differences for the same three colloidal systems in the presence of cytosine arabinoside (not shown).

3-3. Mechanism of Micelle-to-Vesicle Transition in Aqueous Mixtures of Detergent and Phospholipid

In order to examine the different molecular interactions between detergent and phospholipid molecules that are responsible for the vesicle formation from the micelles, we have initially chosen the bile salt-egg PC mixed micellar system as a negatively charged detergent-phospholipid mixture. This is because the phase diagram of the system is well defined and has been extensively reported (Shankland, 1970; Mazer *et al.*, 1980; Claffey and Holzbach, 1981; Carey, 1984;

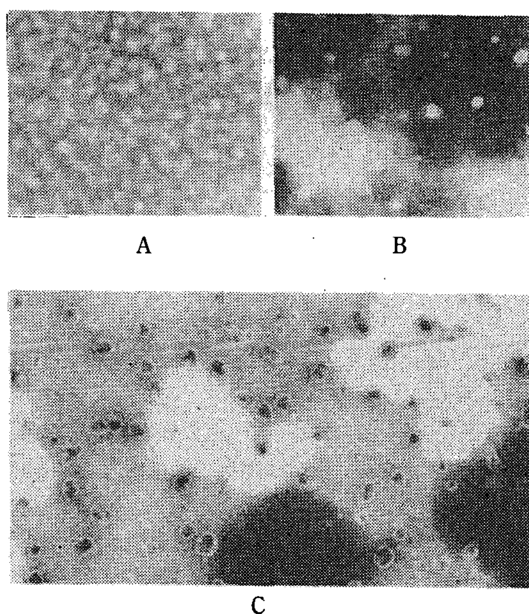


Figure 2—Transmission electron micrographs of GC-egg PC mixed micelles and vesicles (egg PC/GC=0.8) formed in isotonic buffer (pH 7.5), negatively stained with 1% phosphotungstic acid and photographed at 160,000× magnification. Bar equals 100 nm. (A) Mixed micelles (dillution factor of 5), (B) mixed micelles around the phase limit (dilution factor of 8) and (C) mixed vesicles (dilution factor of 10).

Schurtenberger, 1985; Almog *et al.*, 1986; Hjelm *et al.*, 1988 and 1990).

The plot of the apparent mean hydrodynamic diameter ($\bar{d}_h$) of the aggregates vs. the dilution factor is shown in Fig. 3 for five stock solutions having different bile salts but the same egg PC/BS molar ratio (0.8) and total bile salt plus egg PC concentration (50 mg/m*l*). The dilution behavior strongly depends on the type of bile salt. For the deoxycholate (DOC)-egg PC system, not only the maximum of $\bar{d}_h$ is shifted toward much higher dilution factors but the peak of the dilution curve is substantially broadened for the cholate (C)-egg PC system. This suggests that the dihydroxy species has a greater hydrophobic character than the trihydroxy molecule (Armstrong and Carey, 1982), and thus the release rates of the former from the micelles into the intermicellar solution may be much slower than those of the latter. There is a significant shift in the maximum of $\bar{d}_h$ between the conjugated trihydroxy bile salts, taurocholate (TC) and glycocholate (GC): The phase transition occurred at higher dilution in the GC-egg PC system than in the TC-egg PC system. This result can also be explained by the differences in the hydrophobic-hydrophilic properties produced by conjugation (Armstrong and Carey, 1982). However, the phase limit occurred at lower dilution in the C-egg PC system than in the TC- or GC-egg PC systems, which are more hydrohilic. The most likely explanation for this observation is that the size of the hydrophilic group of cholate at the micellar outer surface may be much less than that of either taurocholate or glycocholate, thus promoting the release of cholate molecules from the micelles.

It is of interest that the peak of the dilution curve for the TC/DOC (1:1, mol/mol)-egg PC system falls in between the TC-egg PC and DOC-egg PC system and resembles that of the DOC-egg PC mixture, suggesting that both taurocholate and deoxycholate are dissociating from the aggregates during the transition. Further, the maximum of $\bar{d}_h$ is also observed to be approximately intermediate between that of the TC- and DOC-egg PC systems, as would expected from their hydrophobic character.

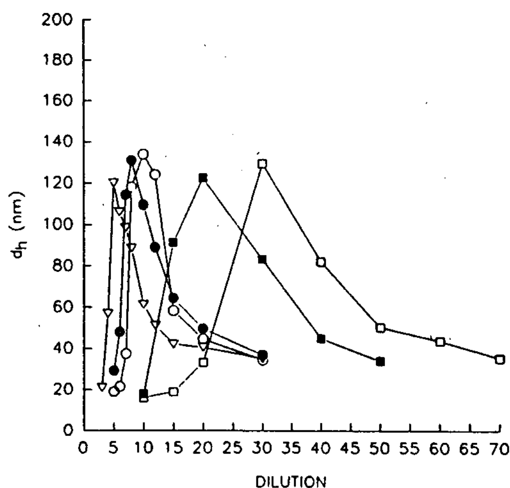


Figure 3—Effect of bile salt species (A) on mean hydrodynamic diameter, $\bar{d}_h$, as a function of the dilution of a mixed micellar stock solution (initial bile salt plus egg PC conc.=50 mg/m*l*) in 0.02 M Tris (pH 7.5) containing 0.15 M NaCl and 0.001 M NaN_3. GC-egg PC(○), TC-egg PC(●), C-egg PC(▽), DOC-egg PC(□), and TC/DOC (1:1, mol/mol)-egg PC(■) at egg PC/BS=0.8 and 23℃. N=3.

3-4. Effect of the Micellar Charge on the Micelle-to-Vesicle Transition

Anionic, cationic, and neutral detergents used were taurocholate, DTAB, and CHAPS and octylglucoside, respectively, while anionic and neutral phospholipids were PA and egg PC, respectively. It is well accepted that cationic amphiphiles confer a positive charge, whereas anionic carry a negative charge. If micelles contain charged phospholipid or detergent, the primary driving force for counterion attraction will be the large surface charge potential rather than the chemical affinity (Seelig *et al.*, 1987). The charged micelles give rise to strong local electrical fields in solution, thus forming the electrical double layer at the micellar surface which has a high charge density. The micellar surface potential

is the electrostatic potential at the micelle-aqueous interface relative to that in the corresponding bulk solution.

In order to test the possibility that charged detergent molecules may not be released from highly charged detergent-phospholipid mixed micelles, negatively charged TC-egg PC micelles (TC is anionic, egg PC zwitterionic) were diluted with medium without counterions such as Na^+, K^+, or Ca^{2+}. As shown in Fig. 4A, there was no change in the micellar size, indicating that vesicle formation does not occur. However, in the presence of Na^+ or Ca^{2+} (data not shown) in dilution medium the micelles grew in size and are transformed into vesicles with a diameter of 32± 4 nm. This result is consistent with the previous reports that increases of the concentrations of Na^+ and Ca^{2+} decreased the magnitude of the potential adjacent to a membrane containing negatively charged phospholipids (Mclaughlin *et al.*, 1981; Alvarez *et al.*, 1983; Brasseur *et al.*, 1984).

To examine the involvement of water structure in the phase transition, since Na^+ and Ca^{2+} are known as water-structure maker (kosmotropes), the micellar stock solution was also diluted with medium containing 0.5 M dextrose only (Fig. 4A). As expected, the micellar size remained unchanged, indicating that the electrostatic effect is a decisive factor in vesicle formation. The results suggest that, as observed previously (Son and Alkan, 1990d), a substantial fraction of high surface charges is neutralized by closely bound counterions that are adsorbed on the micellar surface in the Stern layer (Rathman and Scameborn, 1984). The barrier, the electrical double layer, can be overcome by the neutralization of electrostatic repulsion between the charged hydrophilic group upon binding (adsorption) of oppositely charged counterions.

To further investigate the dependence of the micelle-to-vesicle transition on the counterion binding to the charged micelles, posi-

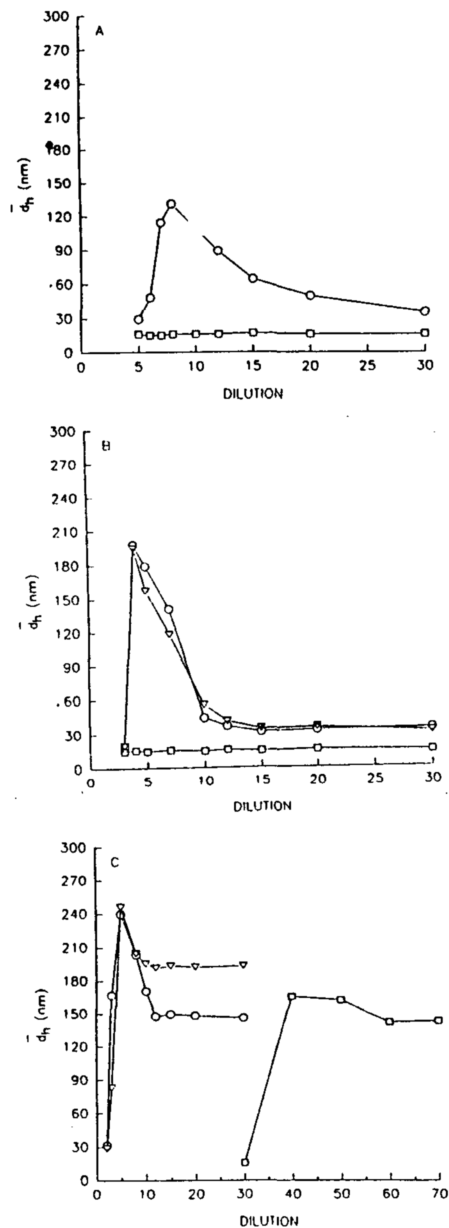


Figure 4—Effect of detergent charges on mean hydrodynamic diameter, $\bar{d}_h$, as a function of the dilution of a micellar stock solution (initial total detergent plus egg PC conc.=50 mg/ml) at 23℃. A: egg PC/TC=0.8 in 0.15 M NaCl at pH 7.5(○) and in water(pH 7.5) or 0.5 M dextrose(□). B: egg PC/DTAB=0.4 in 0.005 M NaI(○), in 0.005 M NaSCN(▽), and in water (pH 7.5) or 0.15 M NaCl, or 0.3 M urea(□). C: egg PC /OG=0.2 in water (pH 7.5) (○), egg PC/CHAPS=0.4 in water(pH 7.5) (▽), egg PC/TC+DTAB(1:1, mol/mol)=0.4 in water(pH 7.5) (□). N=3.

tively charged DTAB-egg PC micelles (DTAB is cationic, egg PC zwitterionic) were diluted in the absence and presence of counterions such as I^-, SCN^-, or Cl^-. As shown in Fig. 9 B, the micellar growth was not observed without counterions as expected, consistent with the result observed in Fig. 9A. When I^- or SCN^- was present in the dilution medium, similar dilution behavior was seen as observed in Figs. 3 and 6, with a lower phase limit. In contrast, the presence of Cl^- had no effect on the micellar size. Only those aqueous anions classified as "water-structure breaker (chaotropes)" (Collins and Washabough, 1985) show significant binding to the positively charged micellar surfaces, which is in reasonable agreement with that reported by Ekmall (1975) and implies that the electrostatic property critical to the ability of an aqueous anion to bind to a micellar surface appears related to its influence on the water structure (Collins and Washabough, 1985). Apparently, the dilution behavior and the final vesicle size (33± 2 nm) of a DTAB-egg PC micellar solution is not influenced by two different chaotropic counterions.

To check the possibility that the chaotropic property of a counterion is more crucial to the vesicle formation than the electrostatic consideration, the dilution behavior of DTAB-egg PC micellar solution was examined in the presence of urea, an aqueous neutral water-structure breaker. Not surprisingly, the micellar size remained unchanged with respect to dilution (Fig. 9B). This suggests that thiocyanate or iodide ion binding occurring in the presence of positively charged micelles was primarily the result of an electrostatic effect, comparable to the previous study that employed conductance and zeta potential measurements (Mclaughlin *et al.*, 1975). Therefore, the binding of chaotropic counterions to fixed cationic charges on the micellar surface causes the electrical neutralization.

It was reported that small aqueous cations (e.g., Na^+, Ca^{2+}, La^{3+}) and anions (e.g., ClO_4^-, I^-, SCN^-) did bind to zwitterionic PC membrane surfaces (Akutsu and Seelig, 1981; Mclaughlin *et al.*, 1975). To further investigate whether the electroneutrality may be achieved by counterion binding to zwitterionic mixed bilayers, neutral octylglucoside-egg PC micelles (octylglucoside is nonionic egg PC zwitterionic) were diluted in the absence of any alkali metal ions or chaotropic anions. As shown in Fig. 9C, the micelle-to-vesicle transition occurs at a lower phase limit and results in a final vesicle size of 148± 4 nm. Further, replacement of octylglucoside by CHAPS (zwitterionic) gives rise to a dilution behavior very similar to octylglucoside micelles, with a final vesicle size of 192± 10 nm. This supports the hypothesis that all or most counterions bind (adsorb) to the surface charges produced by the charged detergent.

Interestingly, when neutral TC/DTAB (1:1, mol/mol)-egg PC micelles were diluted with water (pH 7.5), the transition also occurred as expected. The phase limit occurred at a much higher dilution with a final vesicle size of 146± 6 nm, which is comparable to the size of vesicles spontaneously prepared from aqueous mixtures of simple single-tailed cationic and anionic surfactants acting as double-tailed zwitterionic surfactants (Kaler *et al.*, 1989). The dilution behavior of TC/DTAB (1:1, mol/mol)-egg PC micelles, quite different from either CHAPS- or octyglucoside-egg PC micelles, suggests that specific interaction occur between TC and DTAB, two dissimilar surfactants.

It is also clear that neutral mixed micelles, whether zwitterionic or nonionic, do not privide a barrier to the release of detergent molecules.

3-5. Solubilization of Teniposide in Bile Salt-Egg PC Mixed Micelles and Spontaneous Formation of Teniposide-Containing Liposomes

Free bile acids as well as all alkali metal salts of cholanic acid and its glycine and taurine conjugates (no hydroxyl groups) are ext-

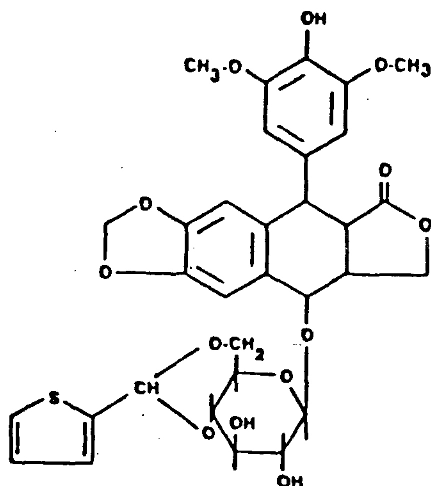


Figure 5—Chemical structure of teniposide.

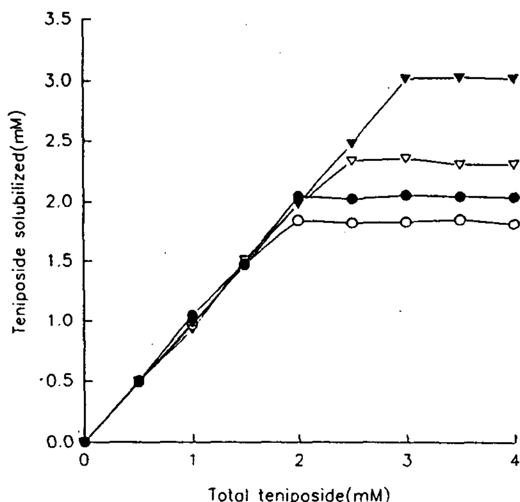


Figure 6—Solubilization of teniposide as a function of the total teniposide in four different bile salts and egg PC solution (PC/BS=1.0, total lipid concentration=50 mg/ml) at 10℃ in Tris/saline buffer(pH 7.5) containing 0.02 M Tris, 0.15 M NaCl and 0.001 M NaN_3. TC-egg PC(○), C-egg PC(●), TCDC-egg PC(▽), TDOC-egg PC(▼). N=3.

remely insoluble in water at temperatures ranging from 0℃ to 100℃ (Javitt and Emerman, 1968). Salts of lithocholic acid (monohydroxy bile salts) are also insoluble at body temperature and highly toxic (Javitt and Emerman, 1968). In contrast, alkali metal salts of di- and tri- hydroxy bile acids and their conjugates are amphiphilic detergent-like

Table III—Solubility of Teniposide at 10℃ in Free or Conjugated Bile Salt-Egg PC Micellar Solution in Tris/Saline Buffer (PC/BS=1.0, Total Bile Salt Plus Egg PC Conc.=50 mg/ml)

Bile salt-egg PC system	Solubility (mM)[a]
TC-egg PC	1.83± 0.04
GC-egg PC	1.88± 0.07
C-egg PC	2.05± 0.04
TCDC-egg PC	2.31± 0.05
GCDC-egg PC	2.25± 0.08
CDC-egg PC	2.33± 0.06
TDOC-egg PC	3.02± 0.06
GDOC-egg PC	3.04± 0.09
DOC-egg PC	3.10± 0.07

[a]Numbers represent the results of three experiments (Mean± S.E.)

molecules which are very soluble in water and possess the capability to solubilize certain quantities of phospholipid bialyers by forming thermodynmaically stabile macromolecular aggregates known as mixed micelles (Carey and Small, 1970). When bile salts are removed, unilamellar phospholipid vesicles are formed with a diameter of 30-100 nm (Son and Alkan, 1989).

Therefore, sodium salts of cholate chenodeoxy-cholate and deoxycholate, and their glycine and taurine conjugates, based on their difference in hydrophilic-hydrophobic balance, were chosen to study the solubilization of teniposide, a practically water-insoluble compound (Fig. 5).

In order to determine the maximum teniposide concentration solubilized in a given system with a single isotropic solution, increased amounts of teniposide were added to each mixed micellar system while the total lipid concentration (50 mg/ml) and the PC/BS ratio (1.0) were kept constant (Fig. 6). The total lipid represents the sum of phospholipid and bile salt. The micellar solubilized teniposide increased and reached a plateau as the total teniposide in the systems increased. The time required to reach the

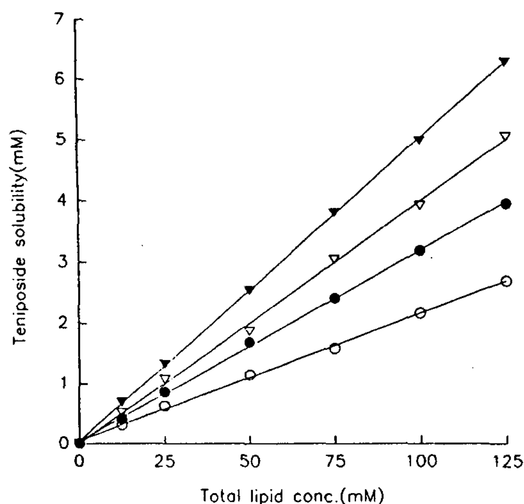


Figure 7—Micellar solubility of teniposide at 10℃ as a function of the total lipid conc.(PC/BS=0.6, 0.9) in Tris/saline buffer. TC-egg PC micelles; 0.6(○), 0.9 (▽), DOC-egg PC micelles; 0.6(●), 0.9(▼). N=3.

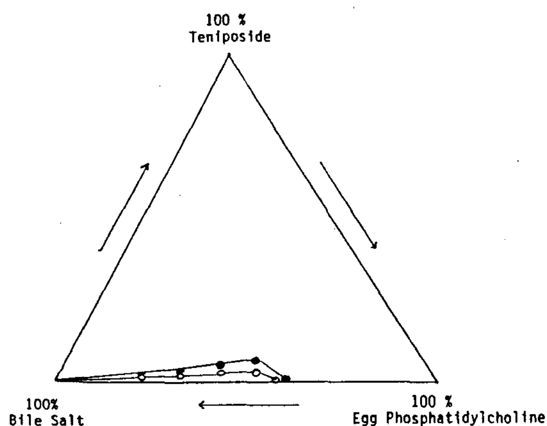
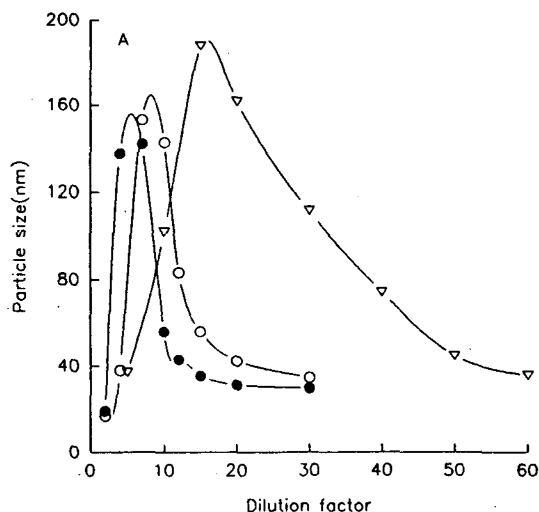


Figure 8—Phase diagram of aqueous taurine-conjugated bile salt-egg PC-teniposide systems. The solid components are expressed in moles per cent for the total lipid conc. of 50 mg/m*l*. Other conditions were 0.02 M Tris (pH 7.5) containing 0.15 M NaCl and 0.001 M NaN_3 at 10℃. Micellar phases are enclosed by solid lines. TC-egg PC(○), TDOC-egg PC(●).



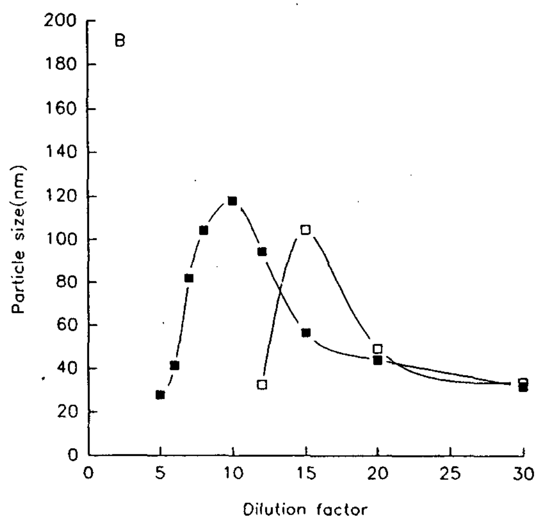


Figure 9—Change in particle size of bile salt-egg PC colloids containing 1.5 mM teniposide, and 50 mg/m*l* of total lipid in Tris/saline buffer as a function of dilution: A. three different bile salts-egg PC system at PC/BS=1.0. TC-egg PC(○), C-egg PC(●), DOC-egg PC(▽), B. TC-egg PC system at PC/BS=0.6(□) and 0.9(■). N=3.

micellar equilibrium in all systems was 24 hrs. The concentration at the plateau can be regarded as the solubility of teniposide at the specified conditions as shown in Fig. 12. The saturation concentrations in various mixed micellar systems are shown in Table III. It was apparent that the solubilizing capacity of dihydroxy bile salts was higher than that of trihydroxy ones; TDOC offered the highest solubility and TC the lowest. There was a large difference in solubilzing capacity between TDOC and TCDC. The glycine or taurine conjugates of trihydroxy bile salts, TC or GC, solubilized to a lesser extent than its corresponding free salts, although the dif-

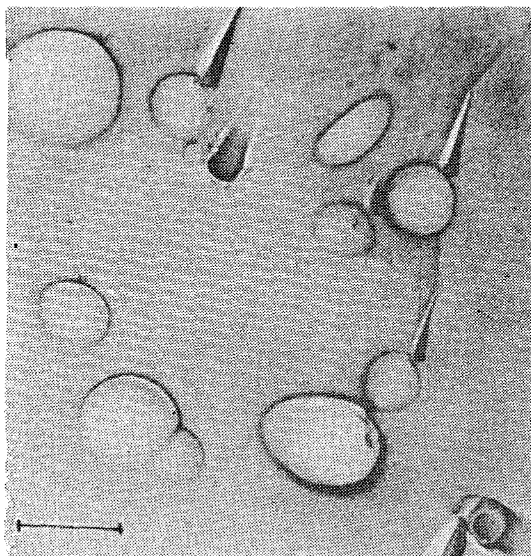


Figure 10—Freeze-fracture electron micrograph of egg PC liposomes (2.05 mg/m*l*) containing teniposide (0.1 mg/m*l*) in the presence of TC (1.28 mg/m*l*) prepared in 0.15 M NaCl, 0.02 M Tris at pH 7.5 with 25%(v/v) glycerol. Bar represents 100 nm.

ference in the solubililizing capacity between them was not large.

However, both free and conjugated dihydroxy species showed a similar solubilizing capacity. It was observed that the aqueous solubility of teniposide in bile salts was negligible (data not shown). On the basis of our observation and other reports (Small, 1968 and 1971) indicating that the lipid solubility was much greater in the bile salt-egg PC combination than in the corresponding bile salt alone, we examined the solubilizing capacity of the mixed miclles with respect to the phospholipid contents in the system (Son and Alkan, 1989b and 1990a).

The effect of the total lipid concentration on the teniposide solubility is shown in Fig. 7 in two micellar systems, TC- and DOC-egg PC, at two PC/BS molar ratios. There was a linear increase in the solubility with an increase in the total lipid concentration up to 125 mg/m*l* at the low and high molar ratios (0.6 and 0.9, respectively). Fig. 8 depicts the phase diagram of teniposide solubility in two different mixed micellar systems. It was clear that teniposide solubility in the micellar phase decreased in proportion to the hydrophobicity of the bile salt.

The dilution of a mixed micellar solution up to the mixed micellar phase limit resulted in a rapid increase in the micellar size (Schurtenberger *et al*., 1985). Fig. 9 shows the effect of dilution on colloidal size of bile salt-egg PC stock solutions. Fig. 9A illustrates the influence of bile salt species on dilution behavior. With increasing hydrophobicity of bile salt from TC to DOC, following three distinct characteristics were found, although the final liposomal sizes in the two systems were comparable: i) the transition occurred more slowly and at higher dilution, ii) the particle sizes at the phase limit increased, and iii) the transition pattern was much broader. It was of interest that the occurrence of the transition at the lower dilution and the larger

Table IV—Teniposide Solubility in Three Different Detergent-Egg PC Systems at 10℃ (Total Detergent Plus Phospholipid Conc.=50 mg/m*l*) in Tris Buffer, pH 7.5 Containing 0.15 M NaCl by Two Different Methods of Mixed Micellar Preparation

Preparation method	Solubility (mM)[a] in mixed micellar system		
	egg PC/TC=0.8	egg PC/CHAPS=0.4	egg PC/OG=0.2
A	1.47± 0.07	ND	2.10± 0.11
B	1.70± 0.07	2.04± 0.10	2.34± 0.12

[a]Numbers represent the result of three experiments (Mean± S.E.).
A: coprecipitation. B: detergent was dissolved in Tris/saline buffer, pH 7.5 prior to its addition to egg PC and teniposide film phase. OG: n-octylglucoside. ND: not determined.

particle size at the phase boundary were observed in C- and TC-egg PC systems compared to DOC-egg PC. As shown in Fig. 9B, the different PC/BS molar ratio with the same total lipid concentration influenced the micelle-to-vesicle transition; the transition peak was shifted toward lower dilution values as the PC/BS molar ratio increased. Freeze-fracture electron micrographs of teniposide-containing vesicles prepared at 3.33 mg/ml total lipid concentration (dilution factor of 15) (Fig. 10) revealed relatively homogenous vesicle populations with a mean size of 62± 14 nm.

Table IV shows the effect of the type of detergent used and the preparation method of mixed micelles on teniposide solubility at 10℃ and in Tris/saline buffer, pH 7.5. TC was selected for this study due to its structural similarity to CHAPS. As expected, CHAPS- and octylglucoside-egg PC systems were found to be better solubilizers than TC-egg PC mixtures. The difference in solubilizing capacity between CHAPS- and octylglucoside-egg PC micelles was not large although much less phospholipid was used in the latter system. It is evident that the preparation method of mixed micelles did not significantly affect the extent of the solubilization, providing the possibility of large scale production.

3-6. Stability of Teniposide in Bile Salt-Egg PC Mixed Micelles and Liposomes

In the previous reports, we have shown that teniposide solubility was dependent not only upon the hydrophobicity of the bile salt, the phospholipid/bile salt molar ratio, total lipid concentration and temperature, but also upon the pH, buffer species, and buffer and NaCl concentration in medium (Son and Alkan, 1990b). In this study, we investigated the stability of teniposide-containing micelles and liposomes with respect to those factors (Son and Alkan, 1990c).

The effect of bile salt species on the precipitation of teniposide in bile salt-egg PC mixed micelles and their corresponding liposo-

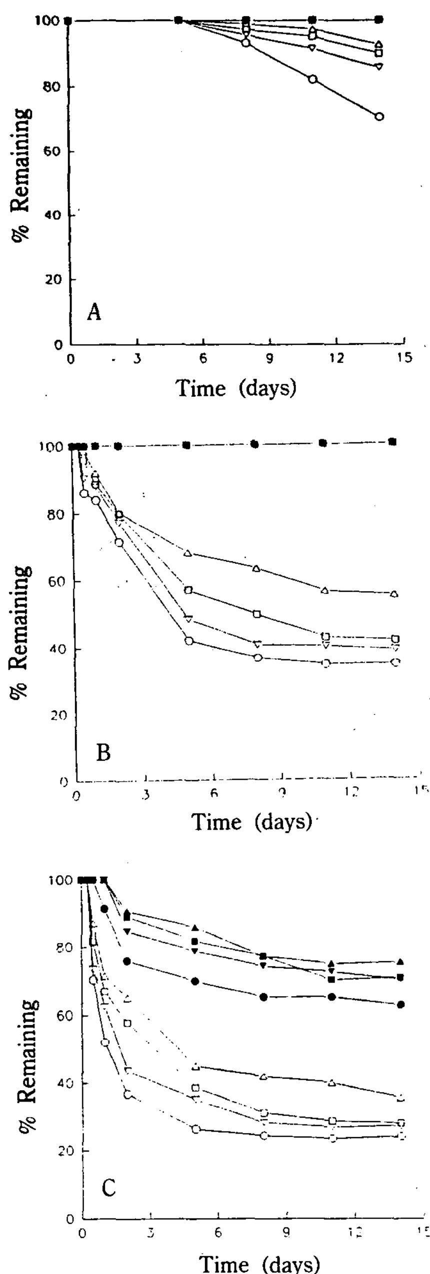


Figure 11—Effect of bile salt species on the percentage of teniposide remaining in bile salts-egg PC mixed micelles (PC/BS=1.0, total lipid conc.=12.5 mg/ml, teniposide conc.=0.375 mM) and their corresponding liposomes (PC/BS=1.0, total lipid conc.=1.67 mg/ml, teniposide conc.=0.05 mM) as a function of time in Tris/saline buffer (pH 7.5) stored at 10℃(A), 23℃(B), and 45℃(C) for 14 days. micelles: GC-PC(○), TC-PC(▽), C-PC(□), DOC-PC(△). vesicles: GC-PC(●), TC-PC(▼), C-PC(■), DOC-PC(▲). N=3.

Table V—Effect of Freeze-Thawing and Freeze-Drying on Percentage of Teniposide Remaining in Mixed Micelles (Egg PC/TC=1.0, Total TC Plus Egg PC Conc.=12.5 mg/m*l*, 0.375 mM Teniposide) during 4 Weeks Storage

Storage condition	micelles			
	1W	2W	3W	4W
freeze-thawing	94.3± 4.7	98.4± 4.3	102.0± 3.5	97.8± 4.7
freeze-drying	97.1± 3.2	95.5± 3.7	98.9± 2.3	102.0± 4.5

Numbers represent the results of three experiments (Mean± S.E.)

mes stored at three different temperatures and in the frozen or freeze-dried forms are shown in Fig. 11A-C and Table V. In general, the hydrophobicity of bile salt in the system was directly proportional to the physical stability of teniposide in both micellar and liposomal formulations, the order being deoxycholate>cholate>taurocholate>glycocholate, for all temperatures studied. At 10℃, the initial precipitation rate of teniposide in the GC-egg PC system was 2.1-fold higher than in the TC-PC system, whereas the difference in the precipitation rate among the TC-, C-, and DOC-egg PC mixed micellar systems was not large (Fig. 11A). At 23℃ and 45℃, teniposide precipitated rapidly during the first 5 days storage and then this precipitation slowed down. After 11 days, there was no further precipitation. The percentage loss in DOC PC micelles was 25-38% in micellar systems and 21-32% in liposomal systems which were less than those observed with three other bile salt systems. The percentage of teniposide loss in the latter systems after 14 days ranged from 25 to 40% (Fig. 11B and C). As expected, liposomes were stable at 10℃ and 23℃ for at least 2 weeks, regardless of what bile salt-egg PC micelles were used to prepare them.

Instability of teniposide in all four liposomal systems at 45℃ was evident because 65-75% of the drug was lost. It was also observed that drug precipitation was greater in micellar systems than in liposomal ones. The increase in stability of teniposide in liposomes was 2.1- to 2.7-fold. However, when all four mixed micellar systems were stored for at least 4 weeks as a frozen or freeze-dried form, the drug did not precipitate (Table V).

Finally we measured the mean hydrodynamic diameters ($\bar{d}_h$) of mixed micelles in buffered saline media with respect to storage conditions and aging. As shown in Table VI, apparent particle sizes remained unchanged at 10℃ or even after freeze-thawing or freeze-drying and rehydration during 4 weeks storage. The average sizes of teniposide-containing micelles (PC/TC=0.8, total lipid conc.=10 mg/m*l*) and liposomes (PC/TC=0.8, total lipid conc.=1.67 mg/m*l*) were found to be 21.3± 3.1 nm and 34.2± 3.8 nm, respectively.

Table VI—Effect of Freeze-Thawing and Freeze-Drying on the Diameter of TC-Egg PC Mixed Micelles (Egg PC/TC=0.8, Total TC Plus Egg PC Conc.=10 mg/m*l*, Teniposide Conc.=0.3 mM) and Liposomes (Egg PC/TC=0.8, Total TC Plus Egg PC Conc.=1.67 mg/m*l*, Teniposide Conc.=0.05 mM) in Tris/Saline Buffer, pH 7.5 during Four Weeks Storage

Storage condition	Diameter (nm)[a]							
	Mixed micelles				Liposomes			
	1W	2W	3W	4W	1W	2W	3W	4W
freeze-thawing	20.4± 2.1	18.6± 2.6	21.7± 3.0	19.6± 1.8	ND	ND	ND	ND
freeze-drying	19.1± 2.2	19.4± 2.0	20.6± 1.8	18.5± 3.1	ND	ND	ND	ND
10℃	20.0± 1.3	19.1± 1.9	18.2± 1.6	21.8± 2.1	32.7± 1.8	34.5± 1.5	36.3± 1.8	34.9± 2.2

[a]Numbers represent the results of three experiments (Mean± S.E.). ND: not determined

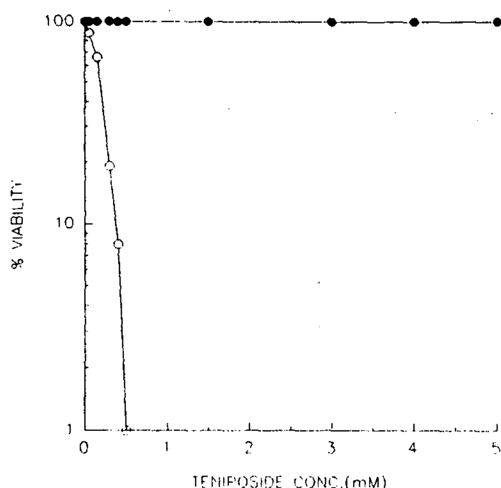


Figure 12—% Viability vs teniposide concentration. the current formulation(●) and the new formulation (○). N=3.

3-7. *In vitro* Evaluation of the Antitumor Potential of Teniposide in the New Formulation

The *in vitro* antitumor activity was plotted as the logarithm of the percentage viability versus teniposide concentration added (Fig. 12). As teniposide in the new aqueous formulation (Table VII) was increased, a gradual decrease in % viability was initially observed. At 300 μM teniposide concentration there were few live cells. To compare the activity of the current formulation (Table VII) with our new one, the studies were conducted at a 10-fold higher concentration. Not surprisingly, almost all tumor cells were still alive at 5.0 mM of teniposide in the current formulation (Fig. 12). This result indicates that teniposide in the nonaqueous vehicle may not be miscible with physiological fluids, thus preventing entry of the drug into cells. We did not observe the difference in cytotoxic activity between two solvents, PBS and 10% serum.

Table VII—Formulations for Teniposide

1. *Current formulation (nonaqueous solution)*	
Benzyl alcohol	0.15 g
Dimethyl acetamide	0.30 g
Castor oil (not polyethoxylated) (Cremophor)	2.50 g
Maleic acid to pH 5.1	q.s.
Absolute alchohol to	4.72 g
Teniposide is supplied in ampules containing 50 mg of the drug in 5 m*l* of a vehicle (teniposide conc.=10 mg/m*l*)	
2. *New formulation (aqueous solution)*	
egg PC-soy PC-TC (1:1:2) or egg PC-soy PC-CHAPS (1:1:5)	
0.02 M PBS, pH 7.5	
10% serum or albumin (4 mg/m*l*)	
5% dextrose	
Total TC plus egg PC conc.=100 mg/m*l*	
Teniposide conc.=7 to 9 mg/m*l*	

4. CONCLUSIONS

1) The quasi-elastic light scattering results obtained along with trapped drug determinations and the electron micrographs of the colloidal samples, support our model that vesicles are spontaneously formed *in vitro* from bile salt-egg PC mixed micelles upon aqueous dilution and that can be an alternative means to sonication in loading drugs.

2) The dilution behavior of bile salt-egg PC micellar solutions depends on bile salt species

3) The micelle-to-vesicle transition occurs when electroneutrality at the micellar surface exists. Therefore, the binding of counterions to charged micelles can make the surface potential neutral.

4) The size of vesicles formed by dilution also depends on the micellar charge.

5) The solubility of teniposide depended upon bile salt species

6) The mixed bile salt-egg PC micelles may be a potential drug carrier system for very lipophilic compounds.

7) Teniposide loss by precipitation from aqueous solutions of bile salt-egg PC mixed micelles depended on the hydrophobicity of bile salt

8) Teniposide-encapsulating micellar sizes were reproducible after freeze-thawing or freeze-drying. Teniposide was much more stabile as liposomes than as micelles: there was no loss of teniposide from liposomes at 10° and 23℃.

9) Freeze-induced particle aggregation and drug leakage in micelles were prevented by the presence of bile salt.

10) The new formulation has an antitumor activity at lower concentrations than the current one.

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