Effects of Lipid Composition on the Transcorneal Penetration of Liposomes Containing Disulfiram, a Potential Anti-cataract Agent, in the Rabbit

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We previously demonstrated that topical treatment with disulfiram (DSF) prevented the development of cataracts in sodium selenite-injected rat pups. In biological systems, DSF is rapidly reduced to diethyldithiocarbamate (DDC), a potent antioxidant. In this study, we investigated the effect of altering the lipid composition of liposomes containing DSF on the transcorneal transit of DDC. Liposomes containing DSF were prepared with various molar ratios of dimyristoylphosphatidylcholine (DMPC), dipalmitoylphosphatidylcholine (DPPC) and cetylpyridinium chloride (CPC) by reverse-phase evaporation. Liposomes with a DMPC to DPPC molar ratio of 5:5, examined by differential scanning calorimetry, had the highest enthalpy of transition and the presence of one molar ratio of CPC further enhanced the enthalpy value. The addition of bovine serum albumin or a homogenate of rabbit cornea to the incubation buffer resulted in the release of DDC, but not DSF from the liposomes. The amount of DDC present in the aqueous humor of rabbit eyes following topical administration increased with increase in DMPC to DPPC ratios and was also enhanced by the addition of CPC to the liposomes. The results of this study suggest that liposome formulations are effective for transcorneal drug delivery of anti-cataract agents such as DSF. DSF in liposomes consisting of DMPC, DPPC, and CPC with a molar ratio of 8:2:1 may be a potential drug formulation for the prevention and/or treatment of cataracts.

Key words liposome; disulfiram; diethyldithiocarbamate; anti-cataract agent; drug delivery system; differential scanning calorimetry

A survey by the World Health Organization reported that 110 million people in the world have poor vision and are at risk of becoming blind. The development of cataracts is the main cause of blindness.¹⁾ There is no effective medical treatment for cataracts. Currently surgical removal with artificial lens replacement is the therapy of choice for cataracts in advanced countries. However, surgery is costly and is not readily available to patients in developing countries where 75% of blindness currently occurs. Developing countries have a high population growth and there is a rapid increase in the number of older people, which is contributing to the upward trend in the incidence of cataracts. For this reason, it is important to develop agents for the prophylactic and therapeutic treatment of this condition.²⁾

Disulfiram (DSF) has been widely used for the treatment of alcohol abuse since it was introduced over 50 years ago.³⁾ Consumption of alcohol while taking a therapeutic dose of disulfiram results in a subjectively unpleasant experience characterized by facial flushing, nausea, tachycardia, rapid respiration, and hypotension (disulfiram-ethanol reaction). This reaction has been ascribed to the inhibition of the enzyme aldehyde dehydrogenase (EC 1.2.13) in the liver, with the result that the consumption of alcohol is promptly followed by the accumulation of toxic amounts of acetaldehyde in the blood.⁴⁾ Administration of DSF results in the rapid production of diethyldithiocarbamate (DDC) which is a product of the catalytic reaction of sulfhydryl groups on proteins, such as albumin, with DSF.⁵⁾ DDC has been shown to inhibit microsomal lipid peroxidation in rat hepatocytes⁶⁾ and both DSF and DDC have been reported to inhibit DNA breakage by agents producing hydroxy radicals.⁷⁾

DDC is unstable in a neutral solution and is not able to

transit through the cornea into the aqueous humor in rabbits.⁸⁾ We postulated that DDC could be delivered to the aqueous humor by instillation of DSF into the eye. DSF would transit the cornea and be converted to DDC. Our preliminary experiments demonstrated that DSF in a liposome formulation had good corneal penetration in the rabbit when compared to other DSF formulations examined, including suspensions.⁸⁾ Moreover, one of the liposome formulations was demonstrated to have anti-cataract effects in sodium selenite-injected rat pups.⁹⁾ In this paper, we examined the effects of altering the lipid composition of the liposomes and the use of cetylpyridinium chloride (CPC) to create a positive charge on the liposome membrane to enhance the transcorneal penetration of DSF-liposomes.

MATERIALS AND METHODS

Animals Male Japanese White rabbits (2.0–2.5 kg body weight) were used. They were housed under standard conditions (12 h/d fluorescent light from 07:00 to 19:00, 25 °C room temperature) and given a commercial diet (CR-3, Clea Japan Inc., Tokyo) and water *ad libitum*. All animal studies were conducted in accordance with the NIH Guidelines on the Care and Use of Animals in Research.

Chemicals DSF was kindly donated by Ouchi Shinko Chemical Industries (Tokyo). Dipalmitoylphosphatidylcholine (DPPC) and dimyristoylphosphatidylcholine (DMPC) were obtained from Nippon Oil and Fats Co., Ltd. (Tokyo). Cetylpyridinium chloride monohydrate (CPC) was purchased from Kanto Chemical Co. (Tokyo). Sodium diethyldithiocarbamate (DDC) and bovine serum albumin (BSA, Fraction V) were purchased from Wako Pure Chemical Industries, Ltd.

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(Osaka, Japan). All other chemicals used were of the highest purity and were commercially available.

Preparation of Liposomes Containing DSF Liposomes containing DSF (DSF-liposomes) were prepared by reverse-phase evaporation with a minor modification.¹⁰⁾ DSF (10 mg), DMPC, DPPC and CPC were dissolved in 20 ml of dichloromethane and these solutions were used as the hydrophobic phase. The molar ratios of DPPC, DMPC and CPC for the DSF-liposomes prepared in this study are listed in Table 1. The sum of the weight of DPPC, DMPC, and CPC was 1.0 g for each formulation. The hydrophobic phase was mixed with 10 ml of phosphate buffered saline (pH 7.4) containing 0.04% methyl and *n*-propyl *p*-hydroxybenzoate and was sonicated under a stream of nitrogen gas using a bath-type sonicator (Branson Ultrasonics Division Emerson-Japan, Kanagawa). After sonication for 10 min at room temperature, the mixture was stirred for 20 min and the dichloromethane was removed under vacuum for at least 30 min at 10–15 Pa. Large unilamellar vesicle liposomes were finally prepared by passing the above mixtures through an Extruder® (Lipex Biomembranes Inc., BC, Canada) with polycarbonate membrane filters (pore sizes of 1.0, 0.6, 0.2 and 0.1 μm , Coster Scientific Corp., MA, U.S.A.), repeated ten times for each membrane. The particle sizes of the liposomes were determined using a laser diffraction scattering particle analyzer, Coulter N4S (Langley Ford Instruments Division of Coulter Electronics of New England, Inc., MA, U.S.A.). The final particle sizes for the liposomes were in the range of 80 to 100 nm; sizes tended to increase with increase in the DMPC content in the liposomes. The particle size distributions are summarized in Table 1.

Differential Scanning Calorimetry (DSC) Ten μl portions of liposome samples were sealed into aluminum sample pans and examined on a differential scanning calorimeter, DSC 8240D (Rigaku Denki Co., Tokyo). Buffer solution served as the reference for the scans. A scan speed of 2 K/min was used for heating and cooling runs over a temperature range of 0 to 80 °C. Purified water was used as a primary standard for temperature and power calibration. Some parameters for the DSC analyses were calculated using a computer analysis system, TAS 300 (Rigaku Denki Co.).

Determination of the ζ -Potential of DSF-Liposomes ζ -Potential of DSF-liposomes was determined using a Lazar Zee Meter, Model 501 (Pen Kem Corp., Ltd., NY, U.S.A.). The liposomes were diluted with 150 vol. of 5 mM potassium phosphate buffer (pH 6.5) and the ζ -potentials were determined at 150 V. The ζ -potential was corrected for temperature variation using the following equation:

$$\zeta\text{-potential (corrected)} = \zeta\text{-potential (observed)} \times (1 - 0.02T)$$

where T was the differential temperature indicated by the apparatus.

Determination of DSF and DDC Levels The amount of DSF in the liposomes and DDC released from DSF-liposomes were determined by an HPLC method. Fifty μl of the sample solution was added to 100 μl of the internal standard solution (5 μg of indomethacin/ml of methanol). Two μl of the mixture was injected into an HPLC instrument, Shimadzu LC-10AD, equipped with a column oven, CTO-6A. The chromatography was performed under the following conditions: a Wakosil-II 3C18 AG (4.6 $\times$ 150 mm, Wako Pure

Table 1. Lipid Compositions and Particle Sizes of DSF-Liposomes

Liposomes (Molar ratio)	Content (mg/10 ml)			Particle size (μm)
	DMPC	DPPC	CPC	
DMPC/DPPC (10:0)	1000	0	0	120 $\pm$ 23
DMPC/DPPC/CPC (10:0:1)	950	0	50	103 $\pm$ 12
DMPC/DPPC (8:2)	787	213	0	94 $\pm$ 11
DMPC/DPPC/CPC (8:2:0.1)	783	212	5	ND
DMPC/DPPC/CPC (8:2:0.3)	775	210	15	ND
DMPC/DPPC/CPC (8:2:0.4)	771	209	20	ND
DMPC/DPPC/CPC (8:2:1)	748	203	49	86 $\pm$ 6
DMPC/DPPC (5:5)	480	520	0	84 $\pm$ 9
DMPC/DPPC/CPC (5:5:1)	457	495	48	79 $\pm$ 12
DMPC/DPPC (2:8)	188	812	0	82 $\pm$ 12
DMPC/DPPC/CPC (2:8:1)	179	774	47	82 $\pm$ 5
DMPC/DPPC (0:10)	0	1000	0	86 $\pm$ 20
DMPC/DPPC/CPC (0:10:1)	0	954	46	87 $\pm$ 14

ND: not determined. Each particle size represents mean $\pm$ distribution from 3–6 experiments.

Chemical Industries, Ltd.) column, mobile phase of 60% acetonitrile containing 0.1% trifluoroacetic acid, flow-rate of 1.0 ml/min, column temperature of 35 °C and a 215 nm wavelength for detection.

Preparation of a Homogenate of Rabbit Corneas Two rabbit corneas (*ca.* 150 mg) were homogenized in 10 ml of Gifford's buffer (pH 7.4) using a Physcotron (Nichion Ikarika Kikai Co., Tokyo). One half of the homogenate was used to examine the release of DDC from DSF-liposomes, and the other half was centrifuged at 43000 $\times g$ for 30 min and the supernatant was harvested. The DDC release experiment was then repeated using the supernatant. The protein and protein SH content in both the homogenate and supernatant were measured.^{11,12)}

DDC Release from DSF-Liposomes The reaction mixture consisted of 0.25 ml of DSF-liposomes diluted 2.5-fold with Gifford's buffer (pH 8.0, 7.4 and 6.5), 0.25 ml of 7% BSA solution dissolved in the same buffer, and rabbit cornea homogenate or supernatant. The reaction was carried out at 35 °C for 0–6 h and was terminated by cooling in an ice bath for at least 3 min. The reaction mixture was ultrafiltered using a Centriscart I (Sartorius AG, Germany, cut-off molecular weight, 10000 dalton). The filtrate was analyzed by HPLC.

DDC released from the DSF-liposomes catalyzed by BSA or by rabbit cornea homogenates or extracts were analyzed by applying a diffusion theory proposed by Higuchi.¹³⁾ Kinetic parameters were calculated using the following equation and an iterative least-squares regression procedure, MULTI¹⁴⁾ using a personal computer, PC-9801RX (NEC Corp., Japan):

$$Q = \sqrt{X \cdot (t - \tau)} \quad (1)$$

where Q is the amount of DDC released from DSF-liposomes at time t , X is the apparent release rate or the complex coefficient, and τ is the lag time until the onset of DDC release.

In Vitro Transcorneal Transit of DDC from DSF-Liposomes The *in vitro* transcorneal transit of DDC from DSF-liposomes was examined using the method of Iwata *et al.*¹⁵⁾ A male rabbit, weighing between 2.5 and 3.0 kg, was killed by injection of a lethal dose of pentobarbital into the mar-

ginal ear vein. The eyes were removed and the corneas were carefully separated from the other ocular tissues. The individual corneas were placed in a methacrylate cell designed for transcorneal transit studies. The side of the chamber with the exterior surface of the cornea was filled with DSF-liposomes. The other side of the chamber (reservoir) contained 10 mM HEPES buffer (pH 7.4) with 136 mM NaCl, 5.3 mM KCl, 1 mM K_2HPO_4 , 1.7 mM $CaCl_2$ and 5.5 mM glucose. The temperature was maintained at 35 °C and the duration of the transit studies was 6 h. Fifty μ l of the sample solution was withdrawn from the reservoir side at the indicated times. The amount of DDC in the samples was determined by HPLC (above). The viability of the corneas was monitored by measurements of thickness and wet weight (no significant changes in thickness or weight were observed over the 6 h period).

The parameters for *in vitro* transcorneal transit of DSF were calculated from the DDC transit data using the following equations¹⁶⁾:

$$\tau = \delta^2 / 6D \quad (2)$$

$$J_c = \frac{K_m \cdot D \cdot C_{lipo}}{\delta} = K_p \cdot C_{lipo} \quad (3)$$

$$Q_t = J_c \cdot A \cdot (t - \tau) \quad (4)$$

where J_c is the DDC transit rate, K_m is the cornea/liposome partition coefficient, D is the diffusion constant within the cornea, τ is the lag time, δ is thickness of the cornea (0.0625 cm, average for 4 rabbits), C_{lipo} is the DSF concentration in the liposomes, Q_t is the total amount of DDC appearing in the reservoir solution at time t , and A is the effective area of cornea (0.79 cm²). J_c and τ were estimated by fitting each transit profile to eq. 4. The penetration coefficient through the cornea, K_p is given by J_c / C_{lipo} . A nonlinear least-squares computer program (MULTI)¹⁴⁾ was employed for the calculation.

In Vivo Transcorneal Transit of DDC from DSF-Liposomes The experiment to study the *in vivo* transcorneal transit of DDC from DSF-liposomes was performed by the method of Meisner *et al.*¹⁷⁾ Fifty μ l of DSF-liposomes was instilled into eyes of rabbits weighing between 2.5 and 3.0 kg. A topical anesthetic was instilled into each eye 3 min before sampling of the aqueous humor. Aqueous humor samples (5 μ l) were removed from the anterior chamber of the eye using a 29-gauge needle) with silicon tubing (inner diameter: 0.5 mm, Fuji Systems Co., Japan) connected to a 25- μ l microsyringe (Ito Corp., Japan). The DDC concentration in the aqueous humor samples was determined by HPLC (above).

The area under the aqueous humor-concentration time curve (*AUC*) and the mean residence time (*MRT*) for transcorneal DDC transit were calculated by the trapezoidal method of Gibaldi and Perrier.¹⁸⁾

Statistical Analysis All values were expressed as the mean $\pm$ S.E.M. of 5–8 experiments. Significance of the mean differences in each experiment was analyzed by Student's *t*-test, and a *p* value of <0.05 was considered significant.

Fig. 1. Calorimetric Heating Curves for DSF-Liposomes Consisting of DMPC and DPPC (Molar Ratio of 8 : 2) with and without CPC

MT, melting point (initial temperature); PW, peak width; PT, temperature at highest peak.

Table 2. Parameters of DSC for DSF-Liposomes with Various Molar Ratios of DMPC and DPPC

Liposomes (Molar ratio)	MT (°C)	PT (°C)	PW (°C)	Enthalpy (kJ/mol)
DMPC/DPPC				
(10 : 0)	23.40	24.15	1.65	-14.2
(8 : 2)	29.74	31.84	4.20	-17.3
(5 : 5)	32.88	36.53	6.27	-24.2
(2 : 8)	41.27	43.73	4.06	-35.0
(0 : 10)	40.65	41.40	1.65	-26.5
DMPC/DPPC/CPC				
(10 : 0 : 1)	25.00	26.05	2.25	-18.1
(8 : 2 : 1)	31.60	33.50	3.90	-22.9
(5 : 5 : 1)	30.60	34.55	6.55	-30.4
(2 : 8 : 1)	40.05	42.70	4.15	-42.3
(0 : 10 : 1)	41.25	41.95	1.60	-28.8

MP, melting point; PT, temperature at peak top; PW, peak width.

RESULTS

DSC for DSF-Liposomes Containing Various Molar Ratios of DMPC and DPPC Figure 1 shows a typical DSC pattern for DSF-liposomes consisting of DMPC and DPPC (molar ratio, 2 : 8) with and without one molar ratio of CPC. The addition of CPC to the liposomes increased the temperature of the highest peak on the DSC. Table 2 shows parameters for DSC analyses of DSF-liposomes consisting of various ratios of DMPC and DPPC. DSF-liposomes consisting of DMPC and DPPC (molar ratio, 2 : 8) had the highest melting point, the highest peak temperature, and the highest enthalpy of transition among the liposomes tested and liposomes having a similar phospholipid composition with CPC had the same characteristics. The highest peak width was observed in DSF-liposomes consisting of DMPC and DPPC (5 : 5) with CPC. The enthalpies of DSF-liposomes with CPC were higher than the liposomes with the same phospholipid composition but without CPC.

Effect of the Molar Ratios of DMPC, DPPC and CPC in DSF-Liposomes on the ζ -Potential The effect of the molar ratios of DMPC, DPPC and CPC in DSF-liposomes on

Fig. 2. Effect of the Addition of CPC on the ζ -Potential of DSF-Liposomes

Each point represents the mean $\pm$ S.E.M. of 5 experiments.

Table 3. Effect of the Molar Ratios of DMPC and DPPC in DSF-Liposomes on the ζ -Potential

Liposomes (Molar ratio)	ζ -Potential (mV)
DMPC/DPPC	
(10:0)	-22.87
(8:2)	-19.81
(5:5)	-14.87
(2:8)	-7.92
(0:10)	-7.84
DMPC/DPPC/CPC	
(10:0:1)	+17.70
(8:2:1)	+23.24
(5:5:1)	+34.93
(2:8:1)	+40.98
(0:10:1)	+39.06

the ζ -potential is summarized in Table 3 and Fig. 2. The ζ -potential was enhanced by increasing the molar ratio of DPPC in the DSF-liposomes. The ζ -potential of all DSF-liposomes containing CPC had a positive value and was 40-45 mV higher than those with corresponding liposome composition but without CPC. For the liposomes consisting of DMPC and DPPC (molar ratio, 8:2), the ζ -potential increased linearly with the increase in molar ratio of CPC in the range from 0 to 1.0 (slope = $42.9 \text{ mV} \cdot (\text{molar ratio of CPC})^{-0.5}$, intercept = -16.0 mV , $r = 0.978$), shown in Fig. 2.

Production of DDC in DSF-Liposome Preparations Catalyzed by BSA and Rabbit Cornea Homogenate Liposomes consisting of only DMPC did not retain DSF for an extended period of time. Almost all of the DSF separated from the liposomes as precipitates within one week following preparation. The liposomes consisting of only DPPC separated into lipid and water phases within 2 weeks following preparation. Therefore, these liposome formulations were not used in the DDC release experiment. Release of DSF and DDC from DSF-liposomes into the incubation medium without BSA was not observed. Therefore, in the experiments, BSA was used both to enhance release and as a catalyst to convert DSF to DDC.⁵⁾ The DDC concentration in the incubation mixture containing DSF-liposomes and BSA increased with incubation time, as shown in Fig. 3. The total

release of DDC at pH 8.0 was about 1.5 times higher than at pH 7.4 (Table 4). The addition of CPC to DSF-liposomes did not affect the release of DDC. The highest DDC release was observed from liposomes containing DMPC and DPPC (molar ratio, 8:2) with and without CPC. Time lags were not observed in the release of DDC from any liposome preparation at pH 8.0. Correlation coefficients (r) for these regression analyses were more than 0.96. DDC was not released from any of the liposome preparations at a pH below 6.5.

The DDC release from the liposome preparation consisting of DMPC, DPPC, and CPC (molar ratio, 8:2:1) at pH 7.4, using the rabbit cornea homogenate and the supernatant is illustrated in Fig. 4. The DDC release using both the corneal homogenate and supernatant increased linearly for the first 5-min ($0.29 \text{ h}^{1/2}$). The DDC concentration using the homogenate plateaued after 10 min ($0.41 \text{ h}^{1/2}$). This non-linearity may have resulted from a component of the homogenate that reacted with the DDC as it was released from the liposomes. The slopes calculated using Higuchi's equation were $63.20 \pm 8.13 \mu\text{M} \cdot \text{h}^{-1/2}$ for the homogenate and $63.31 \pm 3.95 \mu\text{M} \cdot \text{h}^{-1/2}$ for the supernatant, respectively.

Specific activities for the conversion of DSF to DDC using the liposomes containing DMPC, DPPC, and CPC (molar ratio, 8:2:1) were calculated to be $0.554 \pm 0.067 \mu\text{M} \cdot \text{h}^{-1/2} \cdot \text{mg protein}^{-1}$ for BSA, $29.12 \pm 3.73 \mu\text{M} \cdot \text{h}^{-1/2} \cdot \text{mg protein}^{-1}$ for the whole homogenate of rabbit cornea and $102.11 \pm 6.37 \mu\text{M} \cdot \text{h}^{-1/2} \cdot \text{mg protein}^{-1}$ for the supernatant. Total protein SH was $6.82 \pm 0.21 \text{ nmol/mg}$ of protein for BSA, $11.70 \pm 2.88 \text{ nmol/mg}$ protein for the corneal homogenate and $36.47 \pm 1.21 \text{ nmol/mg}$ protein for the supernatant. Specific activities of the conversion, based on protein SH, were $81.23 \pm 6.53 \text{ M} \cdot \text{h}^{-1/2} / \text{mol}$ of protein SH for BSA, $2489 \pm 411 \text{ M} \cdot \text{h}^{-1/2} / \text{mol}$ of protein SH for the corneal homogenate, and $2800 \pm 148 \text{ M} \cdot \text{h}^{-1/2} / \text{mol}$ of protein SH for the supernatant.

The Effect of Molar Ratios of DMPC and DPPC and Addition of CPC in DSF-Liposomes on *in Vitro* Transcorneal Transit The results from the *in vitro* transcorneal transit experiment using rabbit corneas are illustrated in Fig. 5. After approximately 30 min incubation, DDC was observed in the reservoir solution. Table 5 summarizes the pharmacokinetic parameters calculated from the *in vitro* transcorneal transit data. J_c , K_p and K_m increased with an increase of the DMPC to DPPC ratio. Moreover, the addition of CPC to the liposomes increased the parameters, but the increase was not statistically significant.

The Effect of Molar Ratios of DMPC and DPPC and the Addition of CPC in DSF-Liposomes on *in Vivo* Transcorneal Transit The profiles of DDC concentration in the aqueous humor after the instillation of DSF-liposomes into rabbit eyes are illustrated in Fig. 6. The time of peak DDC concentration in the aqueous humor after the instillation of DSF-liposomes without CPC was observed earlier than with CPC. The pharmacokinetic parameters obtained from the *in vivo* transcorneal transit experiment are summarized in Table 6. The bioavailability of DDC in the aqueous humor (AUC) increased with the increased molar ratio of DMPC to DPPC in the DSF-liposomes. The addition of CPC to the DSF-liposomes resulted in an elevated AUC and MRT , but the difference was not statistically significant. Lag times were not affected by variations of the molar ratio of DMPC

Fig. 3. DDC Release from DSF-Liposomes Consisting of Various Ratios of DMPC and DPPC Catalyzed by BSA at pH 7.4 (A) and 8.0 (B)

○, DMPC/DPPC (molar ratio, 8:2); ●, DMPC/DPPC/CPC (molar ratio, 8:2:1); □, DMPC/DPPC (molar ratio, 5:5); ■, DMPC/DPPC/CPC (molar ratio, 5:5:1); △, DMPC/DPPC (molar ratio, 2:8); ▲, DMPC/DPPC/CPC (molar ratio, 2:8:1). Each point represents the mean $\pm$ S.E.M. of 5 experiments.

Table 4. Parameters for DDC Release from DSF-Liposomes with Catalysis by BSA

Liposomes (Molar ratio)	Release at pH 7.4		Release at pH 8.0	
	Slope ($\mu\text{M} \cdot \text{h}^{-1/2}$)	τ (h)	Slope ($\mu\text{M} \cdot \text{h}^{-1/2}$)	τ (h)
DMPC/DPPC				
(8:2)	19.39 $\pm$ 2.34	0.039 $\pm$ 0.011	29.64 $\pm$ 3.02	0.010 $\pm$ 0.004
(5:5)	12.91 $\pm$ 1.95 ^{a)}	0.048 $\pm$ 0.024	19.49 $\pm$ 2.71 ^{a)}	0.001 $\pm$ 0.000
(2:8)	5.25 $\pm$ 1.77 ^{a,b)}	0.188 $\pm$ 0.039	10.12 $\pm$ 0.98 ^{a,b)}	0.022 $\pm$ 0.011
DMPC/DPPC/CPC				
(8:2:1)	18.98 $\pm$ 3.11	0.024 $\pm$ 0.008	27.73 $\pm$ 2.55	0.001 $\pm$ 0.000
(5:5:1)	12.86 $\pm$ 2.03 ^{c)}	0.054 $\pm$ 0.014	16.87 $\pm$ 2.01 ^{c)}	0.000 $\pm$ 0.000
(2:8:1)	5.42 $\pm$ 0.65 ^{c,d)}	0.188 $\pm$ 0.026	8.56 $\pm$ 0.69 ^{c,d)}	0.001 $\pm$ 0.001

a) $p < 0.01$, vs. DMPC/DPPC (8:2); b) $p < 0.01$, vs. DMPC/DPPC (5:5); c) $p < 0.01$, vs. DMPC/DPPC/CPC (8:2:1); d) $p < 0.01$, vs. DMPC/DPPC/CPC (5:5:1). Each value represents the mean $\pm$ S.E.M. of 5 experiments.

Fig. 4. DDC Release from DSF-Liposomes Consisting of DMPC, DPPC, and CPC (Molar Ratio, 8:2:1) by Rabbit Corneal Homogenate at pH 7.4

○, whole corneal homogenate; ●, corneal homogenate supernatant, centrifuged at 43000 $\times g$. Final protein concentrations were 2.17 mg/ml for the corneal homogenate and 0.62 mg/ml for the supernatant of the corneal homogenate.

and DPPC or the addition of CPC.

DISCUSSION

Liposome membranes containing 2 or more synthetic phospholipids are more stable than those containing only one

Fig. 5. *In Vitro* Transcorneal Penetration of DDC from DSF-Liposomes Consisting of Various Molar Ratios of DMPC and DPPC

○, DMPC and DPPC (molar ratio, 8:2); ●, DMPC, DPPC and CPC (molar ratio, 8:2:1); □, DMPC and DPPC (molar ratio, 5:5); ■, DMPC, DPPC and CPC (molar ratio, 5:5:1); △, DMPC and DPPC (molar ratio, 2:8); ▲, DMPC, DPPC and CPC (molar ratio, 2:8:1). Each point represents the mean $\pm$ S.E.M. of 5–8 experiments.

phospholipid.¹⁹⁾ The radius of the outer layer of the liposome membrane is formed by the bilayer of phospholipid and is larger than the radius of the inner layer. DPPC has a longer hydrocarbon side-chain than DMPC and may be aligned in the outer layer of the membrane. This alignment may explain the results of the DSC analysis of the liposomes with a DMPC/DPPC molar ratio of 5:5 that indicated a high enthalpy of transition and the possible formation of stable membranes. A relationship between the release of DDC from DSF-liposomes and the enthalpy of the liposomes was observed. Since the release of DDC from the DSF-liposomes increased with an enlarging molar ratio of DMPC to DPPC, it is thought that the lability of the liposome membranes was the main component affecting release.

CPC is lipophilic and has a positive charge and is added to some ophthalmic preparations as a preservative. We added CPC to the DSF-liposomes to alter the charge on the membrane. CPC was not detected in the ultrafiltrate of liposomes containing CPC, indicating that all of the CPC was incorporated into the liposome membranes, changing the surface charge from negative to positive. In both *in vitro* and *in vivo*

Table 5. Pharmacokinetic Parameters for the *in Vitro* Transcorneal Penetration of DDC Released from DSF-Liposomes with Various Ratios of DMPC and DPPC

Liposomes (Molar ratio)	J_c ($\text{nm} \cdot \text{h}^{-1} \cdot \text{cm}^{-2}$)	K_p ($\times 10^{-2} \text{cm} \cdot \text{h}^{-1}$)	K_m	τ (h)	D ($\times 10^{-3} \text{cm}^2 \cdot \text{h}^{-1}$)
DMPC/DPPC					
(8 : 2)	41.9 ± 11.4	2.01 ± 0.55	0.88 ± 0.39	0.37 ± 0.12	1.98 ± 0.61
(5 : 5)	32.7 ± 12.2^a	1.54 ± 0.39^a	0.76 ± 0.25	0.51 ± 0.29	1.29 ± 0.16^a
(2 : 8)	15.2 ± 6.4^b	0.73 ± 0.38^b	0.16 ± 0.05^b	0.32 ± 0.09	2.80 ± 0.99
DMPC/DPPC/CPC					
(8 : 2 : 1)	51.5 ± 10.8	2.45 ± 0.31	1.14 ± 0.57	0.32 ± 0.22	2.03 ± 0.89
(5 : 5 : 1)	39.2 ± 12.5^c	1.87 ± 0.43^c	0.99 ± 0.61	0.55 ± 0.04	1.18 ± 0.02^c
(2 : 8 : 1)	15.9 ± 4.7^d	0.76 ± 0.08^d	0.31 ± 0.02^d	0.46 ± 0.08	1.56 ± 0.64^c

a) $p < 0.05$, vs. DMPC/DPPC (8 : 2), b) $p < 0.05$, vs. DMPC/DPPC (8 : 2), c) $p < 0.05$, vs. DMPC/DPPC/CPC (8 : 2 : 1), d) $p < 0.01$, vs. DMPC/DPPC/CPC (8 : 2 : 1). Each value represents the mean $\pm$ S.E.M. of 5 experiments.

Fig. 6. *In Vivo* Transcorneal Penetration of DDC from DSF-Liposomes Consisting of Various Molar Ratios of DMPC and DPPC in Rabbits

○, DMPC and DPPC (molar ratio, 8 : 2); ●, DMPC, DPPC and CPC (molar ratio, 8 : 2 : 1); □, DMPC and DPPC (molar ratio, 5 : 5); ■, DMPC, DPPC and CPC (molar ratio, 5 : 5 : 1); △, DMPC and DPPC (molar ratio, 2 : 8); ▲, DMPC, DPPC and CPC (Molar Ratio, 2 : 8 : 1). Each point represents the mean $\pm$ S.E.M. of 6–8 experiments.

Table 6. Pharmacokinetic Parameters for *in Vivo* Transcorneal Penetration of DDC Released from DSF-Liposomes with Various Ratios of DMPC and DPPC

Liposomes (Molar ratio)	AUC ($\mu\text{M} \cdot \text{min}$)	Lag time (min)	MRT (min)
DMPC/DPPC			
(8 : 2)	1011 ± 185	3.32 ± 1.01	62.1 ± 6.2
(5 : 5)	529 ± 131^a	4.94 ± 1.54	57.2 ± 8.5
(2 : 8)	403 ± 163^a	4.21 ± 1.25	50.6 ± 3.3^c
DMPC/DPPC/CPC			
(8 : 2 : 1)	1104 ± 387	4.38 ± 1.59	68.2 ± 11.4
(5 : 5 : 1)	557 ± 106^b	4.95 ± 1.72	64.5 ± 4.0
(2 : 8 : 1)	437 ± 131^b	3.99 ± 1.48	54.0 ± 5.4^d

a) $p < 0.01$, vs. DMPC/DPPC (8 : 2), b) $p < 0.01$, vs. DMPC/DPPC/CPC (8 : 2 : 1), c) $p < 0.05$, vs. DMPC/DPPC (8 : 2), d) $p < 0.05$, vs. DMPC/DPPC/CPC (8 : 2 : 1). Each value represents the mean $\pm$ S.E.M. of 5–8 experiments.

penetration experiments, DSF-liposomes containing CPC had higher DDC transcorneal transit than liposomes without CPC. Schaeffer and Kröhn examined the influence of liposome surface charge on corneal drug penetration.²⁰ Penicillin G flux was found to be greatest with positively charged unilamellar liposomes containing stearylamine, which may reflect a greater binding capacity on the corneal surface. However, it has also been reported that stearylamine was

toxic to many mammalian cells.¹⁹ In this study we used CPC to produce a positive charge on the liposome surface because of the low potential for toxicity.

The dependence of the rate of the reaction between DSF and BSA on pH is not surprising since it is presumably the thiolate form of cysteine that reacts with DSF. Glycylcysteinyglycine would be a better form of cysteine in protein. The cysteine side chain of glycylcysteinyglycine has a pK of 8.0 to 8.7.²¹ If the pK of the thiol in cysteine number 34 in serum albumin was 8.0, a 1.5-fold reduction in the rate constant for the reduction of DSF in DMPC/DPPC (8 : 2) liposomes (from $29.64 \pm 2.55 \mu\text{M} \cdot \text{h}^{-1/2}$ at pH 8.0 to $19.39 \pm 2.34 \mu\text{M} \cdot \text{h}^{-1/2}$ at pH 7.4) may not be observed. This discrepancy may result from the variation of the pK of the cysteine thiol (from 8.5 to 10.1) that results from differences in the state of ionization of the alpha-amino and alpha-carboxyl groups.²² Similar results were obtained for other liposomes. The conversion activity was also measured using the homogenate of rabbit cornea. When the conversion activity was calculated, based on protein SH, the value in the cornea homogenate was approximately 30-fold higher than that of BSA, which suggests that an SH rich protein different from albumin may exist in a cornea.

The cornea consists of 3 layers, the lipophilic epithelium, a water rich stroma, and the endothelium, formed by a monolayer of cells.²³ The proposed mechanism for the penetration of DSF into the cornea and conversion to DDC is illustrated in Fig. 7. DSF-liposomes first attach to the corneal surface and DSF, dissolved in liposomes, transits the corneal epithelium. DSF is converted to DDC in the corneal stroma (the water soluble fraction of the cornea homogenate had the highest catalytic activity). DDC, a hydrophilic compound, could easily pass through the corneal endothelial cell layer and reach the crystalline lens *via* the aqueous humor.

Our preliminary experiments demonstrated that the optimum drug to lipid ratio of the liposome formulation was 10 mg of DSF/g of lipid (1 mg of DSF/ml of liposomes). High DSF/lipid ratios (more than 20 mg of DSF/g of lipid) resulted in precipitates of DSF that separated from the liposomes within one week of preparation. This suggested that the retention of lipophilic drugs such as DSF in liposomes was dependent on the solubility of the drug in the lipid constituent of the liposome.¹⁹

Table 7 shows correlation between enthalpies obtained from DSC analyses, the various parameters for DDC release, and the *in vitro* and *in vivo* transcorneal penetration of DSF-

Fig. 7. Proposed Mechanisms for the Transition of DSF from DSF-Liposomes to Cornea and Conversion to DDC in the Cornea

Table 7. Correlation between the Enthalpy Obtained from DSC Analysis, Various Parameters for DDC Release, and *in Vitro* and *in Vivo* Transcorneal Penetration of DSF-Liposomes

Parameter	Correlation coefficient (<i>r</i>)		
	CPC (–) and (+)	CPC (–)	CPC (+)
Slope at pH 7.4 ^{a)}	0.9217	0.9969	0.9972
Slope at pH 8.0 ^{a)}	0.9482	0.9889	0.9787
<i>J</i> _c ^{b)}	0.8168	0.9987	0.9989
<i>K</i> _p ^{b)}	0.8172	0.9969	0.9972
<i>AUC</i> ^{c)}	0.7836	0.8993	0.8850

CPC (–) and (+), liposomes with and without CPC; CPC (–), liposomes without CPC; CPC (+), liposomes with CPC. *a)* Parameter for DDC release from Table 4, *b)* parameter for *in vitro* penetration from Table 5, *c)* parameter for *in vivo* penetration from Table 6.

liposomes. Because all parameters correlated with liposome enthalpy, liposomes with lower melting points, as indicated by a lower endothermal energy for glass transition of the lipid membrane (a lower enthalpy), may indicate a higher transcorneal transit for DDC.

In conclusion, we examined the correlation between the lipid composition of DSF-liposomes and the transcorneal penetration of the drug in rabbits. DSF-liposomes consisting of a molar ratio of DMPC/DPPC (8:2) had the highest transcorneal penetration. The addition of CPC to the liposomes enhanced penetration. These results suggest that DSF-liposomes with a high transcorneal penetration may be a use-

ful formulation for the medical treatment of cataracts. We are planning to study different DSF-liposome formulations, including freeze-dried preparations, to optimize stability and increase shelf life.

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