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Efficacy of a Liposome Preparation of Anti-inflammatory Steroid as an Ocular Drug-Delivery System

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The efficacy of a liposome preparation on ocular steroid availability was investigated by both tracer studies and investigation of *in vivo* steroid uptake by the cornea. Dexamethasone and its ester derivatives were used as model drugs and aqueous suspensions of each served as control preparations. The liposome preparation containing dexamethasone valerate provided the highest ocular drug levels among the examined preparations. In the case of dexamethasone or dexamethasone palmitate, the liposomal form provided a lower drug level in comparison with the suspension. High esterase activity for dexamethasone valerate was observed in the corneal homogenate supernatant, and most of the steroid taken up after instillation of dexamethasone valerate was metabolized to free alcohol. The corneal dexamethasone level was almost proportional to the concentration of free dexamethasone valerate in the liposome preparation. Only the addition of stearylamine (SA) to the liposomal membrane had an added extra effect on the corneal absorption of dexamethasone valerate.

Keywords —liposome; aqueous suspension; eye drop; dexamethasone; ester derivative; ocular absorption; esterase

Introduction

The topical instillation of eye drops is a convenient and useful therapeutic method. However, the ocular availability of drugs in the form of eye drops is very poor[1] and the resulting drug action is only of short duration.[2] This is due to the rapid clearance of the drops from the precorneal area[3] and the barrier function of the cornea especially to water-soluble drugs.[4] Moreover, the formulation of lipophilic drugs for use in eye drops is very difficult because of their poor water solubility.

The topical instillation of anti-inflammatory steroids is commonly used in the therapy of serious types of ophthalmic inflammation. The topical dosage forms of anti-inflammatory steroids fall into several categories: solutions, aqueous suspensions and ointments. Solutions are without question the most widely used ophthalmic dosage form. However, such drugs should be modified to water-soluble derivatives because of their poor water solubility. It has been reported that unless the corneal epithelium is damaged, dexamethasone sodium phosphate, a water-soluble derivative of dexamethasone, is hardly absorbed by the cornea.[5] Thus, it is doubtful whether such a drug would cure inflammation of the anterior segments. Furthermore, it has also been reported that a higher concentration of fluoromethorone aqueous suspension does not improve the aqueous humor drug concentration, and that its ointment form provides a longer-lasting aqueous humor drug level.[6] However, an ophthalmic ointment is an inconvenient and unpleasant preparation. It cannot be self-administered, and it causes temporarily blurred vision.

Recently, the use of liposomes as an ophthalmic drug device has been advocated to overcome the above problems.[7] Singh and Mezei reported that liposomes increased the ocular tissue concentration of triamcinolone acetonide.[8] However, several studies denying the efficacy of liposomes have also been reported.[9] Although the utility of liposomes would be affected by the physicochemical properties of the drugs they contain and by the lipid composition of the liposomes themselves, systematic studies featuring careful manipulation of these factors have not been performed.

In our previous study,[10] we formulated vari-

ous liposomes containing dexamethasone or its ester derivatives as eye drops, and studied their *in vitro* characterization. In the present study, the efficacy of these liposomes as an ocular system for delivery of water-insoluble drugs was evaluated in rabbit eyes using dexamethasone and its ester derivatives as model drugs.

Materials and Methods

Materials—Dexamethasone (DM) was obtained form Roussel Uclaf (Paris, France), and dexamethasone valerate (DV) and dexamethasone palmitate (DP) were synthesized by the method of Shaw *et al.*[11] [^{3}H]-Dexamethasone and [^{14}C]-dipalmitoyl phosphatidylcholine ([^{14}C]-DPPC) were obtained from Amersham International plc (Buckinghamshire, England) and NEN Research Products (Boston, U.S.A.) respectively, and [^{3}H]-dexamethasone valerate and [^{3}H]-dexamethasone palmitate were synthesized from [^{3}H]-dexamethasone by the method of Shaw *et al.*[11] Egg yolk lecithin (EYL) with a purity in excess of 99% was obtained from Sigma Chemicals (St. Louis, U.S.A.). All other chemicals were of reagent grade and obtained commercially.

Preparation of Liposomes—Liposome preparations were formulated as described in the previous report.[10] Briefly, suitable amounts of lipid and drug were dissolved in chloroform, and the organic solvent was evaporated. The dried lipid film was suspended in isotonic phosphate buffer, pH 7.4, by vortex mixing followed by ultrasonic radiation, usually for 2.5 min, using a probe-type sonicater under nitrogen. After being allowed to stand for a period of 1 h at room temperature, the preparation was filtered through a polycarbonate membrane filter of 1 μm pore size to remove foreign matter such as metal particles formed during sonication. The final drug concentration in each preparation was adjusted to 0.5 mM, which is the lowest concentration in clinical use.

Preparation of Aqueous Suspension—Suitable amounts of Tween 80 and drug were dissolved in methanol, and the solvent was evaporated in a vacuum. The residue was dried and suspended in isotonic phosphate buffer, pH 7.4, by vortex mixing followed by ultrasonic radiation for 5 min using a bath-type sonicater. The final detergent concentration was adjusted to 0.05%.

Tracer Study—The liposome preparation and an aqueous suspension containing tritiated steroid were formulated. Each preparation contained 0.5 mM steroid, and the specific activity of the preparation was 3.6 μCi/μmol of steroid. Each liposome preparation contained 16 mM EYL, and in the case of DV-liposomes, 10 μCi [^{14}C]-DPPC was also added to 5 ml of the preparation in order to label the liposomes. Male New Zealand white rabbits were placed in restraining boxes to minimize movement. The normal upright posture of each animal was maintained at all times. 50 μl doses were administered using a microliter syringe by instillation directly onto the cornea, collecting in the lower cul-de-sac. Animals were then periodically sacrificed by rapid injection of pentobarbital sodium into a marginal ear vein. About 0.2 ml of aqueous humor samples were transferred from the anterior chamber to preweighed counting vials with the aid of a 25-gauge needle attached to a 1 ml disposable syringe. The eyes were immediately enucleated and the cornea, iris and lens were separated, dipped in normal saline and blotted with tissue paper. Each tissue was placed in a separate preweighed vial, which was then reweighed. The tissues were digested by adding 1 ml SOLUENE-350 (Packard). 10 ml of HIONIC-FLUOR (Packard) was added to each vial and the samples were counted in a liquid scintillation counter (TRI-CARB Model 1500, Packard), utilizing a quenching curve prepared by external standardization.

Corneal Metabolic Enzyme Activity—DV or DM solution was prepared in phosphate-buffered saline (PBS) at a concentration of 5 nM. Fresh rabbit cornea was homogenized in 5 ml saline using a Biomixer (Nihonseiki, Tokyo), and 50 μl of the supernatant, obtained by centrifugation at 0 °C, was added to 10 ml of DV or DM solution. After periodic incubation at 37 °C, DV or DM concentration in the incubation medium was determined using a high-performance liquid chromatograph (LC-6A, Shimadzu) equipped with a UV-detector

(SPD-6A, Shimadzu). The stationary phase used was a Nucleosil $10C_{18}$ (Macherey-Nagel)-packed column (4 × 300 mm), and 70% methanol in water containing 1% acetic acid was used as the mobile phase.

Corneal Steroid Absorption——50 μl of a preparation which did not contain any radioactive compound was instilled into the eyes of rabbits using the above method. The animals were sacrificed at 15 min after administration, and each cornea was immediately separated and weighed. After the addition of 5 ml saline, each cornea was homogenized at 0 °C using a Biomixer. The corneal homogenate was extracted with 10 ml ethyl acetate containing fluocinonide as an internal standard. The organic solvent was separated by centrifugation and then evaporated. After addition of 0.5 ml methanol to the residue, extracted steroid was determined by high-performance liquid chromatography (HPLC).

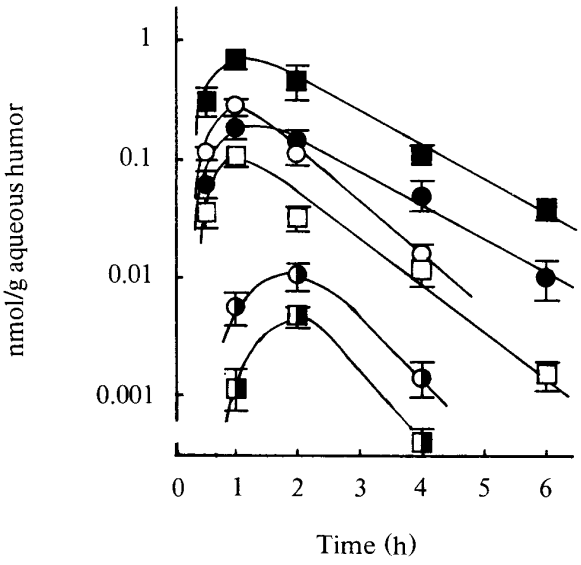

Fig. 1. Aqueous Humor Steroid Concentration after Instillation of Various Preparations Containing [^{3}H]-Dexamethasone or Its Ester Derivatives

Error bars represent standard error of the mean. ○, DM suspension; ●, DV suspension; ◑, DP suspension; □, DM liposomes; ■, DV liposomes; ◨, DP liposomes.

Results and Discussion

Tracer Study

An aqueous humor steroid concentration versus time profile after instillation of the liposome preparation or the aqueous suspension containing DM, DV or DP is shown in Fig. 1. The peak time for each preparation occurred at 1—2 h, and then the aqueous humor level declined according to a first-order process. The steroid concentration after instillation of the DV suspension was almost the same as that obtained with the DM suspension. The maximum concentration after instillation of the DP suspension was much lower than that of DM or DV. The highest aqueous humor steroid level was obtained after the instillation of DV liposome preparation, while in the case of other steroids, liposomes decreased the aqueous humor steroid levels. The liposomes did not alter the peak time or rate of disappearance, but did alter peak level. Accordingly, it is suggested that the liposomes affect only corneal absorption, and do not affect the movement of steroid in the ophthalmic tissues.

The cornea or iris steroid concentration *versus* time profile is illustrated in Fig. 2. The iris steroid level or profile was similar to that of the aqueous humor. The cornea steroid concentration after instillation of each preparation was about 10 times higher than that of the aqueous humor or iris, and the peak time appeared within 30 min. Only a trace of steroid was observed in the lens. A steroid concentration more than 2.5 times higher was obtained and remained for up to 6 h in each ocular tissue with DV liposome preparation compared with that achieved with other preparations. In terms of transcorneal drug penetration, the cornea consists of three basic layers: epithelium, stroma and endothelium.[12] Since the epithelium and endothelium are rich in lipid, they are considered to be a barrier to water-soluble materials. The stroma, on the other hand, is primarily an aqueous structure and provides a barrier to nonpolar compounds. Since corneal steroid concentration was much higher and the peak time was much shorter than that in other tissues, corneal absorption of instilled steroid may be very fast and absorbed steroid may be accumulated in the corneal epithelium and diffuse slowly into the aqueous humor through the corneal stroma.

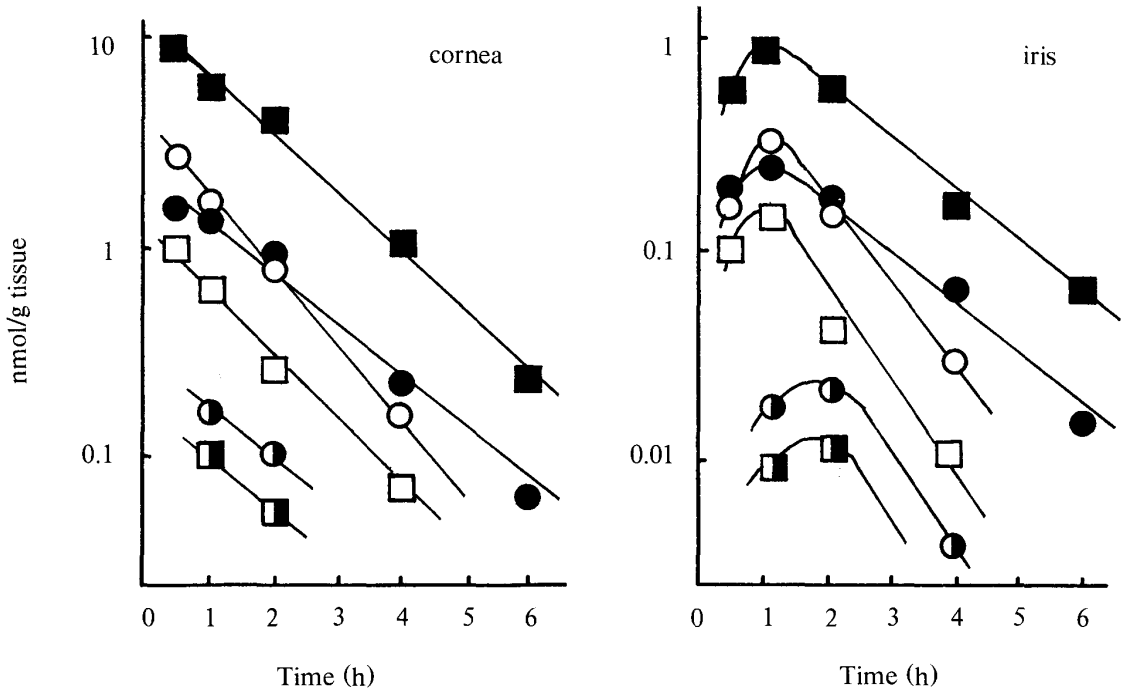

Fig. 2. Cornea or Iris Steroid Concentration after Instillation of the Various Preparations Containing [^{3}H]-Dexamethasone or Its Ester Derivatives

○, DM suspension; ●, DV suspension; ◑, DP suspension; □, DM liposomes; ■, DV liposomes; ◨, DP liposomes.

Though DP was the most lipid-soluble drug, the corneal steroid level after instillation of DP was very low. It is suggested that not only is the diffusion rate of DP through the cornea very slow, but also corneal uptake is very poor. The liposome preparation containing DV provided the highest availability for each ocular tissue. Since the profile of the drug in each tissue was similar to that of the control preparation, the most important role of liposomes may be enhancement of corneal DV absorption.

The corneal steroid or DPPC levels calculated as a percentage of dose are represented in Table I. The corneal absorption of DPPC from the liposome preparation was much lower than that of the steroid. DPPC was not detected in other tissues at all. Various interactions between liposomes and the cell membrane have been suggested,[13] and it was expected that the liposomes might be directly absorbed by the cornea. However, no role of direct absorption of the liposomes in corneal drug absorption is apparent from the results in Table I. This is in agreement with the fact that the liposomes did not improve the corneal availability of DP, which was completely incorporated in the liposomes and was not released at all. Also, the importance of the free drug concentration and drug release from the liposomes in the precorneal area is suggested.

TABLE I. Phosphatidylcholine or Steroid Amount in the Cornea after Administration of DV-Liposome Preparation

Compound	% of dose			
	0.5 h	1.0 h	2.0 h	4.0 h
Phosphatidylcholine [^{14}C]	0.017±0.015	0.014±0.003	0.016±0.004	0.009±0.002
Steroid [^{3}H]	1.42 ±0.50	0.97 ±0.21	0.52 ±0.14	0.16 ±0.03

Corneal Metabolic Enzyme Activity

The liposome preparation containing DV provided the highest drug concentration among the various ocular tissues. However, it was not clear whether absorbed DV had been metabolized. Cheung *et al.* [14] reported that betamethasone valerate was hydrolyzed with the enzyme in mouse skin, and it has also been reported that various ester prodrugs were hydrolyzed with the enzyme in various ocular tissues, especially the cornea.[15] In order to elucidate whether steroids were being metabolized, preliminary work was done using corneal homogenate supernatant. Figure 3 shows the time course for DV and DM when incubated in PBS at 37 °C, with and without the corneal extract. DV rapidly disappeared, and almost the same amount of DM subsequently appeared. A semi-logarithmic plot of the data showed that the disappearance of DV was a first-order phenomenon. Since no degradation was observed unless the corneal extract was added, this disappearance of DV was considered to be due to enzymatic hydrolysis. However, since DM was quite stable when incubated with the corneal extract, it was suggested that DM is accumulated in the cornea after instillation of DV. It has been reported that the effect of ester derivatives of anti-inflammatory steroids on dermal inflammation is stronger than that of the free alcohol.[16] However, it has not been established whether such a drug may act as a prodrug or an antedrug. Recently, Ponec *et al.* [17] investigated the binding affinities of various steroids to glucocorticoid receptor, and suggested that the levels of affinity of valerate ester and free alcohol were almost the same. Since the diffusion of DM in the stroma is considered to be faster than that of DV, which shows a much higher degree of hydrophobicity than DM, this rapid hydrolysis may provide good results in the treatment of inflammation in the anterior chamber.

Corneal Steroid Absorption

Corneal DV or DM concentration, determined by HPLC, at 15 min after instillation of the liposome preparation or aqueous suspension containing DM or DV is shown in Fig. 4. Intact DM was detected in the cornea after instillation of the DM preparation, and the concentration was almost coincident with the results of the tracer study. Since DM was mainly detected instead of unchanged DV, it was considered that absorbed DV was rapidly metabolized to DM. DV may be quickly absorbed by the epithelium

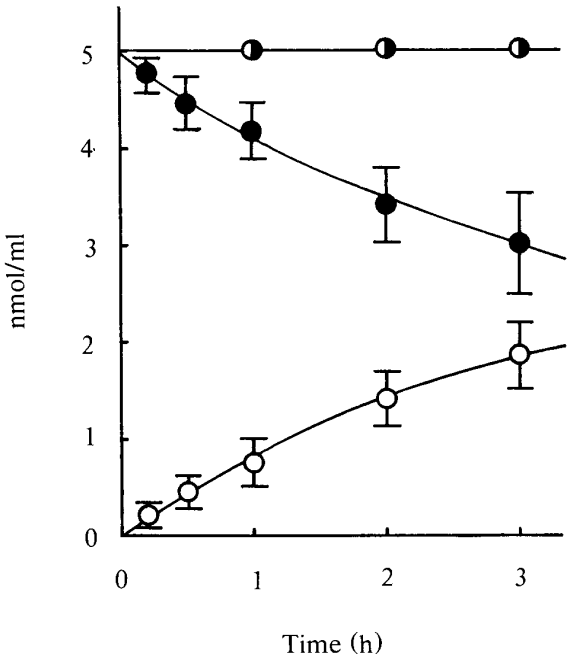

Fig. 3. Hydrolysis of Dexamethasone Valerate by Addition of Corneal Homogenate Supernatant

Each points represent the mean and standard deviation. ◑, enzyme free; ●, disappearance of DV; ○, appearance of DM.

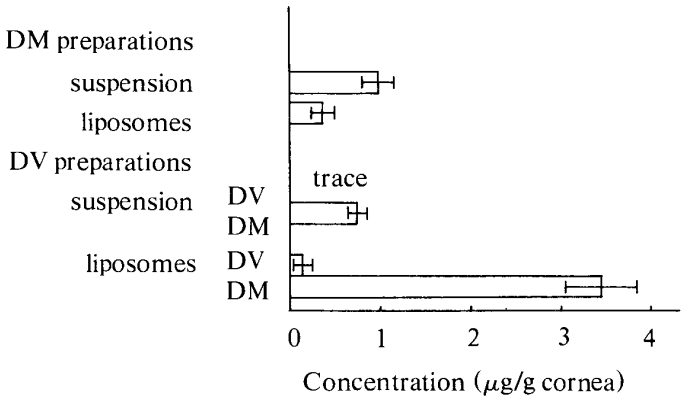

Fig. 4. Corneal Dexamethasone or Dexamethasone Valerate Concentration after Instillation of the Aqueous Suspension or the Liposome Preparation

Error bars represent standard error of the mean.

and metabolized to DM. Then accumulated DM may diffuse slowly through the stroma and transferred to the anterior chamber. It is also suggested that corneal absorption of DV from the various preparations can be estimated by the determination of corneal DM concentration.

To estimate the effect of prescription of liposomes on corneal DV absorption, corneal DM concentration after instillation of various liposome preparations was determined. As shown in Fig. 5, each liposome preparation provided a higher DM concentration than the control preparation. However, the degree of improvement was greatly affected by the prescription of the preparation. In simple liposomes, consisting of only phosphatidylcholine and drug, a higher phosphatidylcholine concentration provided a lower corneal DM concentration, but the period of sonication did not affect corneal DM concentration. These results suggest that corneal DV absorption is related to the free DV concentration but is practically unaffected by the size of the liposomes. Furthermore, the addition of stearylamine (SA) or cholesterol provided a higher corneal DM concentration than that afforded with simple liposomes.

The correlation between cornea DM concentration and free DV concentration in various liposome preparations estimated from the results of Fig. 5 is shown in Fig. 6. A linear correlation appeared to exist for each preparation except for preparation 6. This was in close agreement with the results shown in Table I. Thus it was confirmed that corneal absorption of DV from a liposome preparation may occur from the external aqueous phase. Preparation 6 consisted of positively charged liposomes which contained SA. In this preparation, a higher cornea DM concentration was observed than that estimated from its free DV concentration. It has been reported that *in vitro* liposome uptake by the cornea is greater for positively charged liposomes,[18] and that the introduction of a positive charge on the liposome surface enhances liposome-conjunctiva interaction.[19] This extra effect on the improvement of corneal uptake of DV may be caused by greater interaction of the positively charged liposomes with the corneal or conjunctival surface, or enhancement of corneal permeability by SA.

The liposome preparation containing 16 mM EYL did not improve the ophthalmic availability

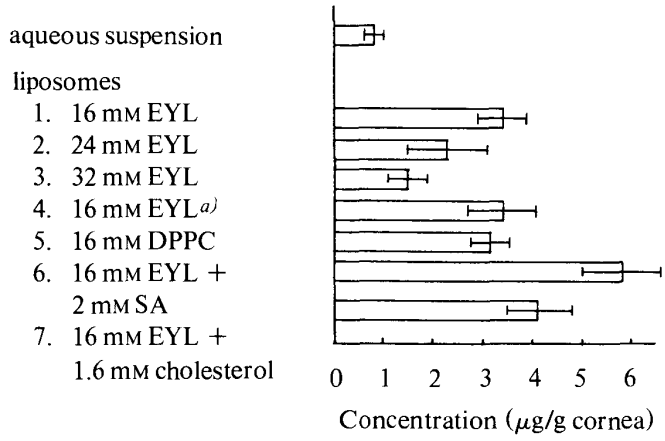

Fig. 5. Corneal Dexamethasone Concentration after Instillation of Various Liposome Preparations Containing 0.5 mM Dexamethasone Valerate

Error bars represent standard error of the mean. *a*) Sonicated for 20 min, others were sonicated for 2.5 min.

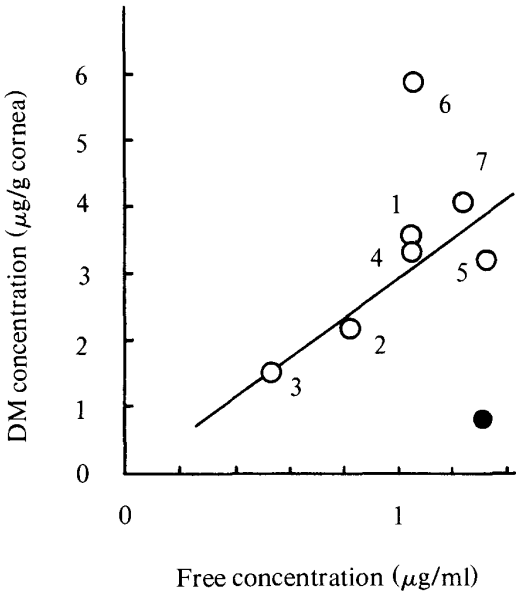

Fig. 6. Correlation between Free Dexamethasone Valerate Concentration in the Various Liposome Preparation and Cornea Dexamethasone Concentration after Instillation to Rabbit Eyes

○, liposome preparation; ●, aqueous suspension.

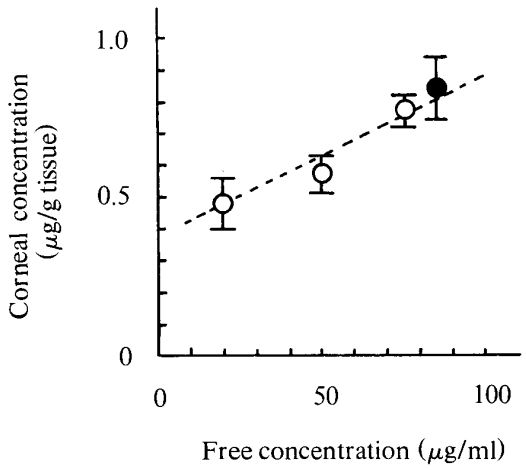

Fig. 7. Correlation between Free Dexamethasone Concentration in the Liposome Preparations and Corneal Dexamethasone Concentration after Instillation to Rabbit Eyes
○, liposome preparation; ●, aqueous suspension.

of DM, as shown in Fig. 4. The free DM concentration in the preparation was 20 μg/ml, which was much lower than its solubility. Consequently, a useful effect of the liposomes on corneal DM absorption might be obtained by increasing the free DM concentration in the preparation. However, as shown in Fig. 7, though DM absorption from the liposome preparation increased when the free concentration was increased by reducing the phosphatidylcholine concentration, DM uptake from the liposome preparation was unable to better that from the aqueous suspension.

With DM, an increase in free concentration caused a considerable decrease in the incorporation ratio in the liposomes. The absence of any good result in DM-liposome preparations was probably due to the relatively low free drug concentration in the preparations or the low incorporation ratio in the liposomes. On the other hand, absorption of DP from the aqueous suspension was very poor, compared with that of DM or DV, and the liposome preparation provided lower DP absorption than the aqueous suspension, as shown in Figs. 1 and 2. These low absorptions may be due to extremely poor water solubility of DP, and the results of our previous report[10] show that neither free DP in the liposome preparation nor release from the liposome has been detected. These results suggest the limitations drug physicochemical properties place on the application of liposomes as an ophthalmic drug device.

Poor ocular drug availability when administered as eye drops is due to rapid clearance of the drops from the precorneal area.[20] Since the liposome preparation provides a high incorporation ratio of DV (in excess of 99%) and release of steroid from liposomes may occur with transition of the equilibrium, as suggested in our previous report,[10] DV may be rapidly released by dilution of the instilled preparation with tear turnover or disappearance of the free drug from the precorneal area with corneal drug absorption or tear turnover. Accordingly, free DV in the precorneal area may be filled up by this rapid release from the liposomes, and may be retained for a longer period. The liposomes themselves would also be expected to be retained for a longer period in the precorneal layer.[21] However, addition of excess phosphatidylcholine may induce a decrease in free drug concentration, and may impair the efficacy of the liposomes.

Though many studies concerning the use of emulsion as a carrier of water-insoluble drugs have been reported,[22] the application of emulsion as an ocular drug device has not been studied. It is hardly expected that the oil droplet of the emulsion can be retained at the precorneal area, and there is neither a lipid digestive enzyme nor a special lipid transport mechanism in the lacrimal gland. Furthermore, it is feared that emulsion causes temporary blurred vision or ocular irritation with high surfactant content. The present study shows that a high incorporation ratio, considerable amount of free drugs and rapid release are required for ophthalmic use of water-insoluble drugs. Since emulsion has high lipid and surfactant contents, it may be difficult to satisfy the above-mentioned conditions. Consequently, emulsion is hardly expected to have good effects on the corneal absorption of water-insoluble drugs.

Since considerable amounts of the intact liposomes may not be absorbed into the cornea, it is not possible to attain an excellent effect of liposomes for water-soluble drugs,[9] which are re-

leased from liposomes according to a first-order process. However, liposomes can be expected to be a good device for delivery of water-insoluble drugs such as DV, the use of which has not previously been possible in eye drops because of their poor water solubility.

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