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Partition Characteristics and Retention of Anti-inflammatory Steroids in Liposomal Ophthalmic Preparations

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Liposome preparations containing dexamethasone or its ester derivatives were formulated as eye drops. Steroids were efficiently incorporated into the liposomes; the incorporation ratio was not affected by the period of sonication or the addition of stearylamine or dicetylphosphate, but was decreased by the addition of cholesterol. The incorporation ratio appears to be governed by partition equilibrium between the lipid membrane and the aqueous phase. A theoretical interpretation of the findings was attempted by using partition theory. No release of dexamethasone palmitate from liposomes was detected, but a small portion of other steroids was rapidly released when the liposome preparation was diluted with a buffer solution. The amount of steroid released from the liposomes may also be governed by the partition equilibrium.

Keywords——eye drop; liposome; anti-inflammatory steroid; incorporation; release profile; partition theory; ultrafiltration; dexamethasone; ester derivative

The topical application of eye drops is a convenient and useful therapeutic method for the treatment of various ocular diseases. An ophthalmic preparation should be a clear aqueous solution to avoid temporary blurred vision and local irritation by particulate foreign matter. However, ocular drug availability as eye drops is very low due to rapid clearance of the drops from the precorneal area, and water-soluble drugs are hardly absorbed by the cornea.[1] For instance, it was reported that corneal absorption of pilocarpine from a solution is terminated within only 5 min following instillation.[2] Thus, an effective and safe ophthalmic preparation appears to be quite difficult to make. Many attempts to overcome these problems have been made using various delivery systems such as ointment,[3] viscous solution,[4] gels,[5] or Ocucert.[6] Recently, the use of liposomes has also been reported,[7] but whether a liposomal preparation will provide any therapeutic advantage remains in question.

The topical instillation of anti-inflammatory steroids is commonly carried out in the therapy of serious ophthalmic inflammation such as iritis or choroiditis. The dosage form should be an aqueous suspension since the steroids are poorly water-soluble or a water soluble derivative solution. Various problems may be encountered in making such a preparation. A higher concentration of a fluoromethorone suspension did not improve the aqueous humor drug concentration,[8] and as the particle size of dexamethasone suspension increased, the drug concentration in the cornea or aqueous humor decreased.[9] Furthermore, Leibowitz *et al.*[10] reported that unless the corneal epithelium is damaged, dexamethasone sodium phosphate, a water soluble derivative of dexamethasone is not absorbed by the cornea.

It thus appears that a more sophisticated preparation which increases the ophthalmic availability and prolongs the interval between doses is necessary to make steroidal therapy safer and more effective. Gregoriadis[11] has suggested the carrier potential of liposomes for lipid-soluble substances, and a number of studies[12] concerning the application of liposomes

containing anti-inflammatory steroid have been reported, mainly for the treatment of rheumatoid arthritis. In the field of ophthalmology, Singh and Mezei[13] reported that liposomes increased the ocular tissue concentration of triamcinolone acetonide. However, the details of the mechanism or factors affecting this improvement in availability have yet to be established.

In the present work, to estimate the usefulness of liposomes for steroid therapy in ophthalmology, we formulated various liposome preparations containing dexamethasone or its ester derivatives as eye drops, and investigated the factors affecting the incorporation of steroids. In addition, the release profiles of drugs from liposomes were studied. The mechanisms involved are discussed.

Materials and Methods

Materials——Dexamethasone (DM) and dexamethasone acetate (DA) were obtained from Roussel Uclaf (Paris, France), and dexamethasone valerate (DV) and dexamethasone palmitate (DP) were synthesized by the method of Show *et al.*[14] Synthesized steroids were identified by thin layer chromatography (TLC), infrared (IR) and nuclear magnetic resonance (NMR) examinations. Egg yolk lecithin (EYL) and dipalmitoyl phosphatidylcholine (DPPC), whose purity was in excess of 99%, were obtained from Sigma Chemicals (St. Louis, U.S.A.). All other chemicals were of reagent grade and were obtained commercially.

Preparation of Liposomes——Suitable amounts of phosphatidylcholine and steroid were dissolved in chloroform, and cholesterol, stearylamine (SA) or dicetylphosphate (DCP) were added as required. The organic solvent was evaporated under vacuum to a thin lipid film. The film was dried and suspended in pH 7.4 isotonic phosphate buffer by Vortex mixing followed by ultrasonic radiation, usually for 2.5 min, using an Ohtake 5202 sonicator under nitrogen. The liposomes containing DPPC were prepared at 70 °C, and the others were prepared at 0 °C. After standing for a period of 1 h at room temperature, the liposome suspension was filtered with polycarbonate membrane filter of 1 μm pore size (Nuclepore Corp., Pleasanton, U.S.A.) to remove foreign matters such as metal particle formed during sonication.

Evaluation of Liposome–Steroids Interaction——The liposome preparations were ultrafiltered using a micropartition system, MPS-1 (Amicon, Mass, U.S.A.), and the steroid concentration in the preparation (C_T) and that in the ultrafiltrate (C_W) were assayed by high performance liquid chromatography (HPLC). The steroid concentration before membrane filtration (C_S) was also determined. Recovery of the steroid in the membrane filtrate (FR) was,

$$\mathrm{FR}(\%)=\left(\frac{C_T}{C_S}\right)\cdot 100 \tag{1}$$

C_W was considered to be free steroid concentration, since phosphatidylcholine was not detected in the ultrafiltrate. Thus, the incorporation ratio in the liposomes (IR) was,

$$\mathrm{IR}(\%)=\left(\frac{C_T-C_W}{C_T}\right)\cdot 100 \tag{2}$$

and the distribution ratio of the steroid (DR) was,

$$\mathrm{DR}=\frac{C_T-C_W}{C_W} \tag{3}$$

Dexamethasone Uptake into the Liposomes——The EYL liposomes free of the steroids were prepared by the previous method, and DM solution was prepared in ethanol at a concentration of 25 mM. Then 50 μl of the DM solution were added to 10 ml of the empty liposome suspensions, and the final DM concentration was adjusted to 50 μg/ml. After periodic incubation at 37 °C, the free DM concentration was estimated by ultrafiltration (MPS-1).

Release Experiment——Following preincubation at 37 °C for 1 h, the liposome preparation was diluted with phosphate buffered saline (pH 7.4) prewarmed at 37 °C, and immediately after dilution, it was incubated at 37 °C. The initial steroid concentration in the preparation (C_T) and that in the aqueous phase (C_W) were determined before dilution. The free steroid concentration was monitored by periodical ultrafiltration of samples. Retention of the steroid in the liposomes was,

$$\text{remaining ratio}=\frac{C_T/N-C_N}{(C_T-C_W)/N} \tag{4}$$

$$\text{percent remaining}=(\text{remaining ratio})\cdot 100$$

where N is the dilution ratio and C_N is the steroid concentration in the ultrafiltrate of the samples.

Analytical Methods——The steroids were determined by HPLC on an apparatus (LC-6A, Shimadzu, Kyoto) equipped with a variable wavelength ultraviolet (UV) detector (SPD-6A, Shimadzu). The stationary phase used was a Nucleosil $10C_{18}$ (Macherey-Nagel, Dueren, Germany) packed column (4 × 300 mm), and 95% methanol in water for DP or 70% methanol in water containing 1% acetic acid for other steroids was used as the mobile phase, at a flow rate of 1.0 ml/min. Chromatograms of the standard solution were obtained and calibration lines were constructed on the basis of peak area measurements. Phosphatidylcholine was assayed using a Wako Phospholipid B-Test (Wako Chemicals, Osaka) based on a colorimetric reaction. No disturbance of the colorimetric reaction due to the presence of the steroids was detected.

Results and Discussion

Factors Affecting Steroid Incorporation into Liposomes

The physicochemical properties of the steroids used in this study are shown in Table I. The ester derivatives showed higher partition coefficients to chloroform and lower solubilities in the buffer than DM. DP was almost insoluble in the buffer and the partition coefficient was so high that it could not be determined exactly. Other steroids showed amphiphilic properties. However, DM, which had the highest hydrophilicity, showed a maximum solubility of only 80 μg/ml in the buffer solution.

Though the interaction of drugs and liposomes has been evaluated by gel filtration[15] or ultracentrifugation,[16] some problems were encountered when using these methods in the preliminary experiments. Steroid leakage during gel filtration was unavoidable, and a portion of the liposomes remained in the supernatant following ultracentrifugation. On the other hand, there were no problems such as adsorption, release of the steroid or appearance of phosphatidylcholine in the filtrate when the micropartition system was used for estimating

TABLE I. Physicochemical Properties of Dexamethasone and Its Ester Derivatives

Compound	Molecular weight	Melting point (°C)	PC_{CHCl_3} [a]	Solubility[b] (μg/ml)
Dexamethasone	392.45	262—264	9.0	80.5
Dexamethasone acetate	452.52	226—229	657	29.6
Dexamethasone valerate	493.58	214—217	8780	1.3
Dexamethasone palmitate	630.88	55—60	—	0.052

a) Partition coefficient between chloroform and water. *b*) Solubility in pH 7.4 buffer solution at 37 C.

TABLE II. Effect of Sonication Time on the Incorporation of Various Steroids in Liposomes

Sonication time (min)	Distribution volume (%)[a]	Filtration ratio (%)[b] ± S.D.				Incorporation ratio (%) ± S.D.			
		DM	DA	DV	DP	DM	DA	DV	DP
2.5	1.5	101.3 ±2.6	99.6 ±1.5	100.2 ±0.9	101.6 ±1.5	90.5 ±1.3	94.7 ±0.3	99.5 ±0.0	(100)
5.0	1.3	101.5 ±2.0	100.6 ±1.6	98.0 ±0.9	100.8 ±1.5	91.8 ±0.8	95.5 ±0.2	99.5 ±0.1	(100)
10.0	1.0	100.7 ±0.4	102.1 ±2.9	99.3 ±2.7	99.3 ±0.6	92.2 ±0.3	96.0 ±0.6	99.6 ±0.0	(100)
20.0	0.7	100.5 ±2.0	101.4 ±2.1	99.5 ±0.3	100.5 ±0.4	92.0 ±1.5	95.7 ±0.2	99.6 ±0.0	(100)

a) Determined by the method of Oku *et al.*[17] *b*) Recovery of the steroids in the filtrate after polycarbonate membrane (1 μm) filtration.

free steroid concentration in the liposome suspension.

The effect of the sonication period on the incorporation of various steroids is shown in Table II. The concentrations of EYL and the steroid were fixed at 16 and 0.5 mM, respectively, in the following experiments. No steroid loss during membrane filtration was observed in any system. The incorporation ratio increased with increasing lipophilicity of the steroids. Even DM, which showed the lowest lipophilicity, provided a high incorporation ratio of more than 90% and DP was confirmed to be completely incorporated into the liposomes. From a practical standpoint, the amount of the steroids in the inner aqueous phase may be small enough to be neglected, because of the low free concentration of steroids and the small distribution volume of the liposomes. Liposome diameter appeared to become smaller with increasing time of sonication, since the turbidity and distribution volume decreased. However, the incorporation ratio was not affected by the sonication period. Thus, the steroids may be incorporated mainly into the lipid bilayer, and the incorporation ratio may depend on the properties of the lipid constituting the liposome membrane and not on the shape or size of the liposomes.

The effects of cholesterol on free steroid concentration are shown in Fig. 1. The free concentrations of various steroids except DP increased with the addition of cholesterol. This is in agreement with the previous reports that the penetration of hydrocortisone decreases upon addition of cholesterol to DPPC monolayer systems,[18] and the addition of cholesterol to lipid dispersion decreases the uptake of hydrocortisone and other steroids by the membrane.[19] Even when cholesterol was added to the liposomal membrane, free DP could

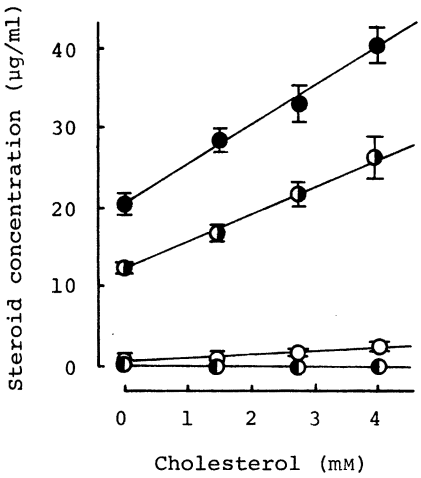

Fig. 1. Effect of Cholesterol on Free Steroid Concentration in the Liposome Preparation

Each preparation contained 16 mM EYL and 0.5 mM steroid. ●, DM; ◑, DA; ○, DV; ◐, DP.

TABLE III. Effect of Lipid Composition on the Incorporation of Steroids in Liposomes

Composition of liposomes	Incorporation ratio (%) ± S.D.			
	DM	DA	DV	DP
EYL : steroid = 16 : 0.5	90.5 ± 1.3	94.7 ± 0.3	99.5 ± 0.0	(100)
EYL : SA : steroid = 16 : 2 : 0.5	91.2 ± 0.5	94.5 ± 0.2	99.5 ± 0.0	(100)
EYL : DCP : steroid = 16 : 2 : 0.5	90.5 ± 1.1	94.9 ± 0.6	99.5 ± 0.1	(100)
DOPC : steroid = 16 : 0.5	92.1 ± 0.4	95.8 ± 0.6	99.7 ± 0.0	(100)
DPPC : steroid = 16 : 0.5[a)]	78.5 ± 1.4	91.0 ± 0.2	99.3 ± 0.0	(100)

a) Prepared at 70 C. Others were prepared at 0 C.

not detected. Cleary and Zats[18] suggested that since hydrocortisone has a polar group at each end of the molecule, a horizontal orientation is favored, so that all polar groups remain hydrated. On the other hand, Fildes and Oliver[20] suggested that cortisone-21-palmitate is anchored in the phospholipid bilayer by an acyl side chain. Thus, it is likely that the incorporation mode of DP differs from those of other steroids.

Table III shows the incorporation ratio of steroids into various liposomes. DP was completely incorporated in all cases. As far as other steroids were concerned, the incorporation ratio was unaffected by the addition of SA or DCP, which rendered the liposomal surface electrically charged. The ratio was affected by the species of phosphatidylcholine. It is considered that the addition of SA or DCP may not induce any transition in the inner structure of the lipid membrane, while the membrane structure consisting of saturated phosphatidylcholine is so rigid that the number of binding sites of amphiphilic steroids in the liposomes may be reduced.

Mechanism of Incorporation

The influence of additional DM concentration on liposome–steroid interaction is illustrated in Fig. 2. The liposomes consisted of EYL and DM, and the concentration of EYL was fixed at 16 mM. The free DM concentration increased linearly with additional DM concentration up to 2 mM, but further addition had no effect. This plateau level coincided with the solubility of DM in the buffer. When free DM became saturated, the recovery of DM in the membrane filtrate dropped while EYL was entirely recovered in the filtrate. This steroid loss may be caused by crystallization. However, the incorporation ratio of DM was always 90% irrespective of additional DM concentration. This suggests the existence of a partition equilibrium of the steroid between the liposomes and the aqueous phase.

To confirm this hypothesis, the uptake of DM by empty liposomes was investigated. Free DM concentration after the addition of DM to the liposome suspension is shown in Fig. 3. It was much lower than that following the addition of DM only 5 min later. This steady-state

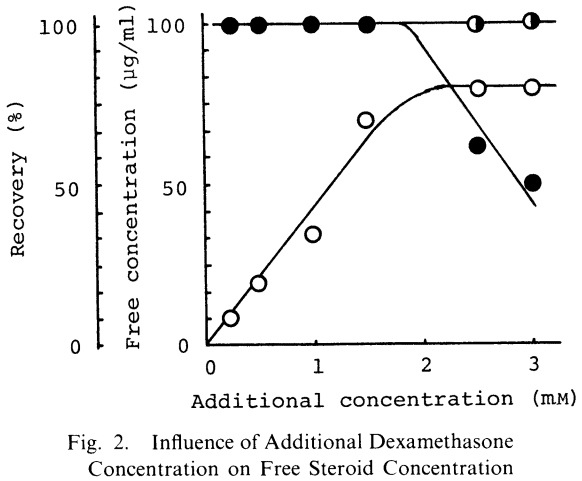

Fig. 2. Influence of Additional Dexamethasone Concentration on Free Steroid Concentration and Recovery of Phosphatidylcholine or Dexamethasone in the Polycarbonate Membrane Filtrate

○, free dexamethasone concentration; ●, dexamethasone recovery in polycarbonate membrane filtrate; ◑, phosphatidylcholine recovery in polycarbonate membrane filtrate.

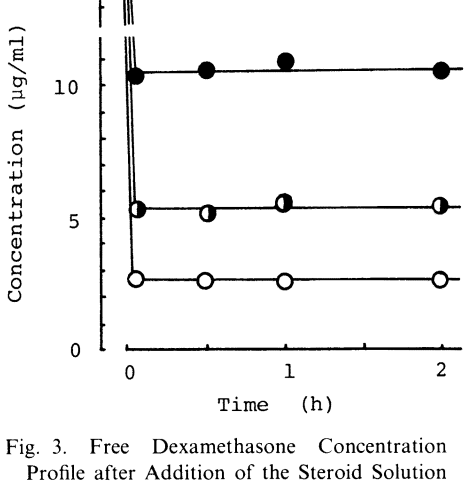

Fig. 3. Free Dexamethasone Concentration Profile after Addition of the Steroid Solution to the Various Empty Liposome Suspensions

○, 32 mM EYL; ◑, 16 mM EYL; ●, 8 mM EYL.

free concentration decreased with increase in EYL concentration. Thus, additional DM may be rapidly taken up by the liposomes, possibly by the partition of DM to the liposomal membrane.

If a partition equilibrium is established, the partition coefficient (PC) may be defined as,

$$PC=\frac{C_L}{C_W}=\frac{X_L \cdot V_W}{X_W \cdot V_L}=\text{constant} \tag{5}$$

where C_L is the steroid concentration in the lipid membrane, C_W is that in the aqueous phase, X_L and X_W are the steroid amounts in the liposomes and the aqueous phase, respectively, and V_L and V_W are the volume ratios of the liposomes and the aqueous phase, respectively. The DR is,

$$DR=\frac{X_L}{X_W}=\frac{C_L \cdot V_L}{C_W \cdot V_W}=PC \cdot \left(\frac{V_L}{1-V_L}\right) \tag{6}$$

Since $V_L \ll 1$ and $V_L = W_L/A$ where W_L is the lipid concentration in the liposome suspension and A is specific gravity of the lipid, Eq. 6 becomes,

$$DR=\left(\frac{PC}{A}\right) \cdot W_L \tag{7}$$

The correlation of the distribution ratio in the liposome preparations determined by Eq. 3 with the additional concentration of EYL is shown in Fig. 4. The distribution ratio of each steroid increased linearly with EYL concentration, which is in agreement with the theoretical relation between DR and W_L in Eq. 7.[21] It is thus confirmed that the incorporation ratio of steroids such as DM, DA or DV may be governed by partition equilibrium. The partition coefficient of DA and that of DV are 2 times and 20 times greater than that of DM, respectively, from the slopes of the lines in Fig. 3.

As far as DP was concerned, neither free steroid nor crystal could be observed in any

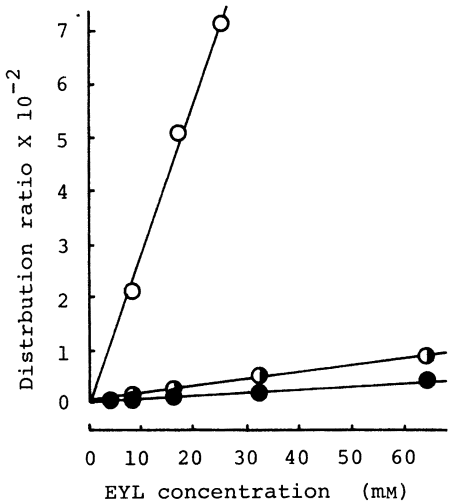

Fig. 4. Relationship between Distribution Ratio of the Steroid and Phosphatidylcholine Concentration in the Liposome Preparation

Each liposome preparation consisted of EYL and steroid, and contained 0.5 mM steroid. ●, DM; ◑, DA; ○, DV.

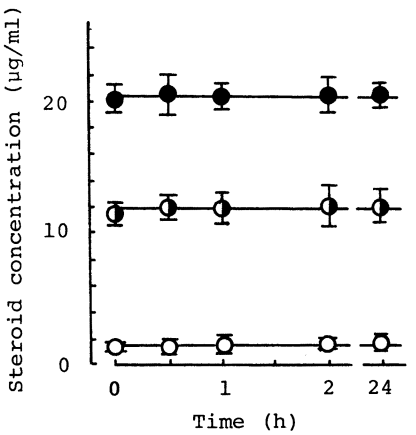

Fig. 5. Free Steroid Concentration in the Liposome Preparations after Incubation at 37 °C without Dilution

Each preparation contained 16 mM EYL and 0.5 mM steroid. ●, DM; ◑, DA; ○, DV.

system. DP was considered to be completely incorporated into the liposomes as one of the components constituting the membrane structure, such as cholesterol.

Release of Steroids from Liposomes

Arrowsmith *et al.*[22] reported that the *in vitro* release of steroids from liposomes proceeds by first-order kinetics once an initial phase of rapid loss is terminated, but the details of the initial phase were not discussed.

The free steroid concentration in the liposome preparation following incubation at 37 °C is shown in Fig. 5 to confirm the stability of steroid incorporation. The free concentration of each steroid was quite stable for 24 h.

The release profiles of the steroids following dilution are shown in Fig. 6. A part of the steroids was rapidly released within 5 min and after that no further release occurred within 24 h. The percentage of steroids remaining in the liposomes at the steady state increased with the lipophilicity of the steroid, and decreased with increasing dilution ratio. Considering that this rapid release may be caused by transition of the equilibrium as a result of dilution, we carried out the following theoretical analysis.

The volume ratio of the lipid (V_L) was so small that the volume ratio of the aqueous phase (V_W) could be considered as unity. From Eq. 5, the steroid amount in the liposomes (X_L) is,

$$X_L = PC \cdot (X_T - X_L) \cdot \frac{V_L}{1 - V_L} \tag{8}$$

where X_T is the total amount in the liposome preparation. Since V_L is sufficiently small, Eq. 8 becomes,

$$X_L = \frac{PC \cdot V_L}{1 + PC \cdot V_L} \cdot X_T \tag{9}$$

Thus, the steroid amounts in the liposomes before dilution, X_0, and after dilution, X_N, are,

$$X_0 = \frac{PC \cdot V_L}{1 + PC \cdot V_L} \cdot X_T \tag{10}$$

$$X_N = \frac{PC \cdot (V_L/N)}{1 + PC \cdot (V_L/N)} \cdot X_T \tag{11}$$

where N is the dilution ratio. The remaining ratio in the liposomes, R_N, is,

$$R_N = \frac{X_N}{X_0} = \frac{1 + PC \cdot V_L}{N + PC \cdot V_L}$$

$$\frac{1}{R_N} = \frac{1}{1 + PC \cdot V_L} N + \frac{PC \cdot V_L}{1 + PC \cdot V_L} \tag{12}$$

The correlation between the reciprocal value of the remaining ratio in the steady state determined experimentally by applying Eq. 4, and the dilution ratio is shown in Fig. 7. A linear correlation appears to exist for each steroid. This is in close agreement with the theoretical relation in Eq. 12. Thus it is confirmed that release of the steroid from the liposomes may occur during transition of the equilibrium. As long as the preparation is not diluted or the free steroid concentration is not decreased, the steroid will not be released from the liposomes.

Drugs instilled as eye drops rapidly disappear from the precorneal area.[23] If it is possible for liposomes to be retained at the precorneal area for a reasonable period of time, they might serve as a good drug vehicle. However, even if liposomes remain at the precorneal area, drugs

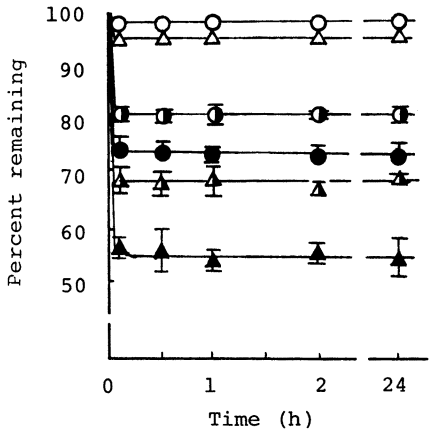

Fig. 6. *In Vitro* Release Profile of the Steroids from the Liposomes after Dilution with PBS (pH 7.4)

●, DM (×5); ◑, DA (×5); ○, DV (×5); ▲, DM (×10); ◭, DA (×10); △, DV (×10).

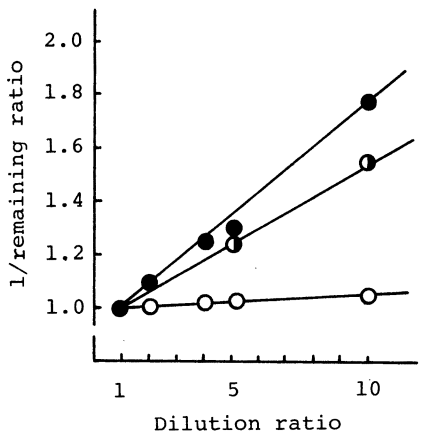

Fig. 7. Relationship between Reciprocal Value of the Remaining Ratio of the Steroids in the Liposomes after Dilution and the Dilution Ratio

●, DM; ◑, DA; ○, DV.

slowly released from the liposomes may not be absorbed by the ocular tissue due to rapid clearance by tear flow. The liposome preparation containing DP, which is completely incorporated into liposomes and not released, may not be satisfactory, unless the liposomes can be directly taken up into the cornea or DP can be transferred directly from the liposome membrane to the cell membrane, as in the case of cholesterol.[24] It is doubtful whether a considerable amount of drug can be absorbed in such a manner. Incorporated steroids except DP may be released by dilution of the preparation with tears or clearance of free steroids. Free steroids in the precorneal layer may possibly be retained for a longer period. Besides, a liposome preparation, in contrast to other drug delivery systems, can be easily self-administered by patients. Accordingly, liposome preparations containing steroids such as DM, DA or DV could prove to be useful ophthalmic delivery systems for treatment of various eye inflammations.

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