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Effects of topically applied liposomes on disposition of epinephrine and inulin in the albino rabbit eye

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Summary

By virtue of the biocompatibility of their constituent phospholipids and of their ability to influence cell membrane permeability, liposomes are an attractive system for topical ocular drug delivery. The objective of this study was to investigate whether the ocular disposition of epinephrine and inulin in the albino rabbit was similarly affected following their encapsulation in multilamellar liposomes. Drug concentrations in tears, conjunctiva, cornea, iris plus ciliary body and aqueous humor were monitored at 30 min post-instillation of various preparations of each drug using radiotracer techniques. Liposomal drug entrapment was found to have opposite effects on the corneal and conjunctival absorption of epinephrine and inulin, epinephrine absorption was reduced by 50% whereas inulin absorption was increased 10 times. Quite unexpectedly, although inulin was detected in the uveal tract, none of it was detected in the aqueous humor when presented in liposomal form. These preliminary data suggest that while the corneal and conjunctival absorption of a drug can be modified by its entrapment in liposomes, its disposition in the intraocular tissues is unlikely to be controlled entirely by liposomes, since few, if any, of the liposomes that may be absorbed are expected to maintain their integrity while permeating the cornea.

Introduction

The eye possesses a number of rather efficient barriers which protect it from the influence of drugs. The cornea skillfully limits the access of most drugs found in the

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tear pool, polar water-soluble drugs in particular, to the internal eye. But its ability to do so can be compromised by increasing the lipophilicity of the compound through prodrug derivatization, as is the case with epinephrine (Wei et al., 1978). While this prodrug approach shows promise in promoting uptake of low molecular weight compounds by the cornea, it may not be as successful with higher molecular weight substances such as substance P antagonists, epidermal growth factor and interferon. In this instance, liposomes may be an alternative mode for delivering these potential therapeutic agents.

Liposomes are membrane-like vesicles consisting of one or more concentric lipid bilayers alternating with aqueous compartments (Kimelberg and Mayhew, 1978). They have been investigated for their ability to enhance the bioavailability of drugs from the oral (Patel and Ryman, 1977), intramuscular (Arakawa et al., 1975), and, more recently, the dermal (Mezei and Gulasekharam, 1980) and ocular (Smolin et al., 1981; Schaeffer and Krohn, 1982) routes of administration. Their potential in topical ocular drug delivery has been brought to the forefront of medical attention by two recent studies. Smolin et al. (1981) reported that in the treatment of acute and chronic herpetic keratitis in albino rabbits, idoxuridine entrapped in liposomes was more effective than a comparable therapeutic regimen of unentrapped idoxuridine. Schaeffer et al. (1982) reported that the transcorneal flux of penicillin-G and indoxole doubled when these drugs were presented to the corneal surfaces in liposomal form. These promising results notwithstanding, it is necessary to assess the potential as well as limitations of liposomes in ocular drug delivery by defining the conditions under which liposomes would enhance ocular drug absorption and by elucidating the mechanisms by which liposomes interact with the two major absorptive surfaces of the eye—cornea and conjunctiva.

The objective of this research was to investigate whether the ocular disposition of epinephrine and inulin in the albino rabbit was similarly affected following their encapsulation in multilamellar liposomes consisting of phosphatidylcholine and cholesterol. Both compounds penetrate the cornea poorly (Keller et al., 1980; Wei et al., 1978), although they differ widely in their molecular structure, molecular weight and aqueous solubility characteristics. Epinephrine, a well known antiglaucoma drug, was chosen as a model compound for low molecular weight compounds, whereas inulin, a non-therapeutic agent, was chosen as a model compound for high molecular weight compounds such as substance P antagonists, epidermal growth factor and interferon. Inulin was chosen over other high molecular compounds primarily because it is water-soluble, non-ionizable, pharmacologically inert, and not known to be metabolized during and following transport. The concentration of each compound in tears, conjunctiva, iris plus ciliary body and aqueous humor was monitored at 30 min post-dosing. This time point corresponds to the time at which drug absorption from the tear pool across the cornea into the aqueous humor following solution instillation typically ceases (Sieg and Robinson, 1976). The drug concentration achieved in these ocular tissues or fluids after administration of liposome-entrapped drugs was compared to that achieved under 3 control conditions: free drug, free drug mixed with empty liposomes immediately prior to topical instillation, and free drug administered 15 min after topical instillation of empty

liposomes. These controls were necessary to evaluate whether complete or partial obstruction of the drainage apparatus by liposomes, or alteration of the epithelial surfaces of the cornea and conjunctiva by liposomes, was responsible for alteration in drug concentration as a result of liposomal entrapment of the drug. The second control (free drug mixed with empty liposomes) served an additional purpose of ensuring that enhancement in drug uptake by liposomes was not due solely to drug adsorbed on the exterior surfaces of liposomes. Likewise, the third control was designed to determine if the effect that liposomes might have on the corneal surfaces was time-dependent thereby altering drug uptake. The data of the present study suggested that neither of these mechanisms was responsible for the alteration in ocular uptake of liposome-entrapped epinephrine and inulin. However, the influence of charge and size of liposomes on the disposition of epinephrine and inulin, as reported for penicillin-G (Schaeffer and Krohn, 1982), was not evaluated in the present study.

Materials

Type VE L-α-phosphatidylcholine, cholesterol and epinephrine-HCl were obtained from Sigma Chemicals (St. Louis, MO). Inulin was obtained from Pfaltz and Bauer, (Stamford, CT). [^{14}C]Cholesterol, spec. act. 59.4 mCi · mmol^{-1}; [^{3}H]epinephrine-HCl, spec. act. 13.5 Ci · mmol^{-1}; [^{3}H]inulin, spec. act. 225.2 mCi · g^{-1}, were obtained from New England Nuclear (Boston, MA). Except for [^{3}H]epinephrine-HCl, all compounds were used as received.

Methods

(1) Preparation of dosing preparations

Epinephrine-HCl solutions

A 0.5% epinephrine-HCl solution was prepared fresh in 0.01 N acetate buffer at pH 4 containing 0.2% $NaHSO_3$, hereafter to be referred to as acetate buffer, which was found to prevent oxidation of epinephrine-HCl over the time course of the experiment. To this solution was added [^{3}H]epinephrine-HCl, prepurified by vacuum evaporation to remove the tritiated solvent in which the radioactive epinephrine was dissolved (Chrai and Robinson, 1974), such that each milliliter of the final solution contained 0.25 mCi of radioactive material.

In those experiments involving the concurrent administration of free drug and empty liposomes, a 1.25% solution of epinephrine-HCl was prepared. The solution was mixed with a liposomal preparation containing [^{14}C]cholesterol, prepared as outlined in a subsequent section, in a ratio of 2 : 3 prior to topical instillation onto rabbit eyes.

Inulin solutions

A 0.5% solution containing both non-radioactive and tritiated inulin was prepared fresh in acetate buffer, such that each milliliter of the final solution contained 0.25 mCi of [^{3}H]inulin. The tritiated inulin was supplied as a powder, and as such was not purified. The molecular weight distribution of inulin (5000–5500) was verified by the supplier (New England Nuclear, Boston, MA) using gel permeation chromatography on Sephadex LH-20 with 0.3% NaCl solution as the mobile phase. In addition, there was no evidence of tritium exchange over the duration of elution. Mixtures containing inulin and empty liposomes were prepared as for epinephrine-HCl.

Liposomal suspensions

Liposomes were prepared using a modified sonication method (Kimelberg and Mayhew, 1978). L-α-Phosphatidylcholine (7.50 mg), cholesterol (1.11 mg) and [^{14}C]cholesterol (1.5675 μCi) were dissolved in 500 μl of chloroform contained in a 5 ml round-bottomed flask, and the solution was evaporated to form a thin film on the walls of the flask using a flash evaporator (Buchler Instruments, Fort Lee, NJ). A 5% solution of epinephrine-HCl or inulin in acetate buffer, 750 μl, was added to the round-bottomed flask together with 20 solid glass beads (3 mm in diameter). This step was followed by dispersing the thin film of phosphatidylcholine and cholesterol into the aqueous solution by vortexing at low speed for 10 min. The suspension that resulted was quantitatively transferred to another 5 ml round-bottomed flask, allowed to equilibrate at room temperature for 1 h, and subjected to sonication for 5 min, under nitrogen, in a Bransonic 52 sonicating bath (Branson Instruments, Shelton, CT) partially filled with an ice-water mixture. These conditions tend to favor multilamellar liposomes. The crude liposomal preparation was immediately transferred to a polyallomer centrifuge tube (Curtin Matheson Scientific, Fountain Valley, CA). After equilibration at room temperature for 30 min the preparation was centrifuged at 15,000 rpm for 15 min in a Sorvall RC2-B centrifuge (DuPont Instruments, Newton, CT). The supernate, which contained unentrapped drug,was decanted and saved for counting for radioactivity. The pellet was resuspended, with vortexing, in 1 ml of acetate buffer followed by centrifugation to remove additional unentrapped material. This resuspension–centrifugation procedure was repeated for a total of 3 times, with the supernate saved at the end of each cycle for counting for radioactivity. The final pellet was resuspended, with vortexing, in 550 μl of acetate buffer to yield a liposomal preparation which then was instilled to the rabbit eye. Each milliliter of this preparation contained 0.25 mCi of tritiated epinephrine-HCl or inulin. Control, empty liposomal preparations were also made according to the procedure just outlined.

In preliminary experiments the gel filtration procedure (Huang, 1969) commonly used to separate unentrapped drug from liposomes was found to be unsatisfactory. Consequently, the resuspension–centrifugation procedure was employed. It was found that repeating this procedure beyond 3 times did not significantly increase the removal of unentrapped material, as judged by constancy in radioactive counts in the supernates. Mass balance of total radioactivity was achieved, and from the

counts due to tritiated drug, the *apparent* entrapment efficiency was calculated to be 5.48 ± 2.62 ($n = 6$) for epinephrine-HCl and 2.93 ± 0.44 ($n = 9$) for inulin. These values were within the typical range of entrapment efficiency reported for low molecular weight compounds (Fendler and Romero, 1976; Forssen and Tokes, 1979; Schaeffer and Krohn, 1982).

(2) Drug concentration in tears, conjunctiva, cornea, aqueous humor and iris-ciliary body at 30 min post-instillation of preparations containing epinephrine-HCl or inulin

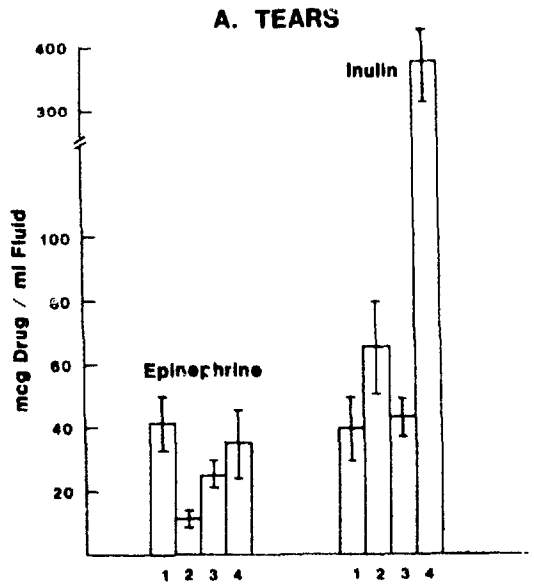
Male, albino rabbits (ABC Rabbitry, Pomona, CA), weighing 2.3–2.6 kg, were used throughout the study. During the experiments, all rabbits were kept in restraining boxes in a normal upright posture. Both eyes of the rabbits were used. Twenty-five microliters of a dosing preparation containing either epinephrine-HCl or inulin were instilled directly onto the cornea of the rabbit, collecting in the cul-de-sac. During instillation, the upper lid was slightly raised and the lower lid was pulled slightly away from the globe. The lids were immediately returned to their normal position after instillation. During one of the experiments, the rabbit eye was predosed with 25 μl of empty liposomal suspension 15 min prior to instillation of solutions of either epinephrine-HCl or inulin.

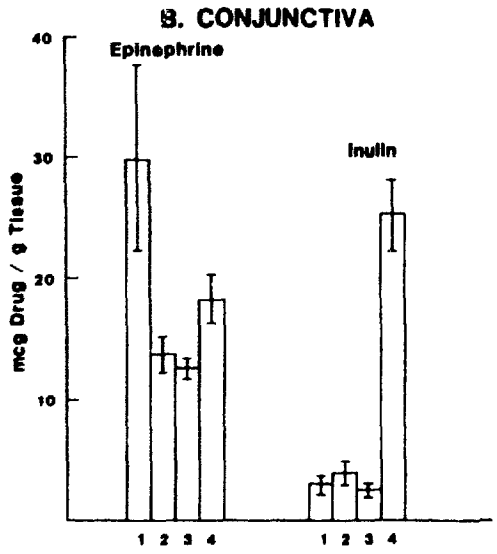
Approximately 5 s prior to sacrificing the rabbit, 1 μl of tears was collected using a disposable glass capillary pipet (Curtin Matheson Scientific, Fountain Valley, CA). Pipets containing tear samples were transferred to vials (BioVials, Beckman, Irvine, CA) containing 4 ml of prerefrigerated scintillation cocktail (Aquasol-2, New England Nuclear, Boston, MA), and counted in a liquid scintillation spectrometer (Beckman Model 7500, Irvine, CA) after 24 h of storage in the dark. The presence of glass capillaries in the scintillation cocktail was found not to alter the counting efficiency or affect the results in a significant way.

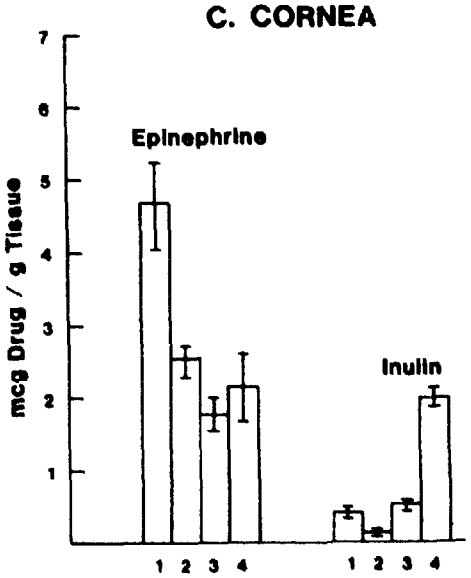
At 30 min post-dosing, the rabbit was sacrificed by marginal ear vein injection of a 30% sodium phenobarbital solution. Its conjunctival and corneal surfaces were thoroughly rinsed with normal saline and blotted dry to remove residual radioactivity. Between 150 and 200 μl of aqueous humor were aspirated using a 27-gauge $\times$ 0.5 in. needle attached to a 1 ml tuberculin syringe, and the conjunctiva, cornea, iris plus ciliary body [1] were removed sequentially using a surgical scalpel.

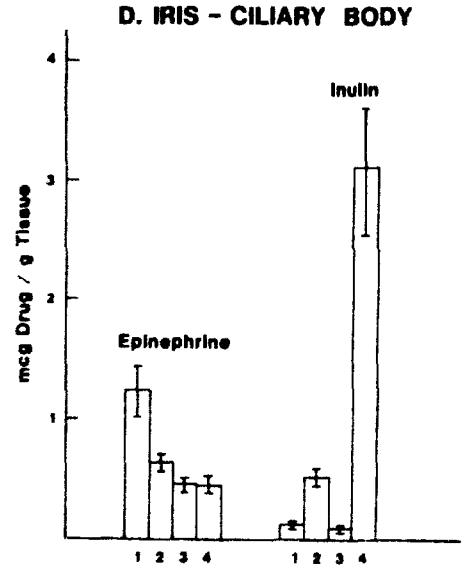
The aqueous humor samples were transferred to vials (BioVials, Beckman, Irvine, CA) containing 4 ml of prerefrigerated scintillation cocktail (Aquasol-2, New England Nuclear, Boston, MA). Each of the tissue samples was digested at 55°C for 18 h in 1.5 ml of a tissue solubilizer (Protosol, New England Nuclear, Boston, MA) contained in a glass scintillation vial (Curtin Matheson Scientific, Fountain Valley, CA) followed by decolorization in 100 μl of hydrogen peroxide and addition of 10 ml of scintillation cocktail (Econofluor, New England Nuclear, Boston, MA). All samples were stored in the dark for 24 h prior to counting in the liquid scintillation spectrometer (Beckman Model 7500, Irvine, CA). After correcting for background

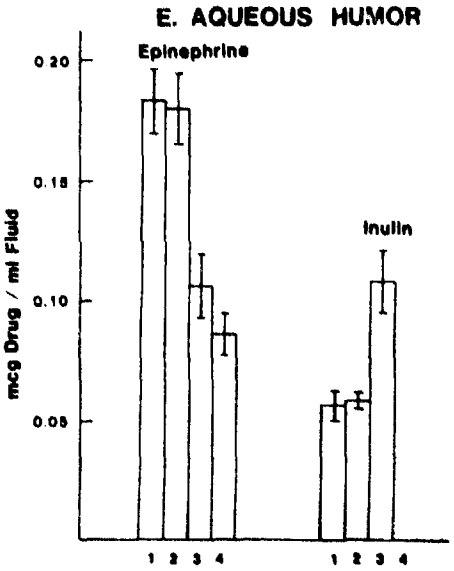
[1] The iris and ciliary body were removed as one structure and hereafter will be referred to as iris-ciliary body


A. TEARS
mcg Drug / ml Fluid
400
300
100
80
60
40
20
Epinephrine
Inulin
1 2 3 4
1 2 3 4


B. CONJUNCTIVA
mcg Drug / g Tissue
40
30
20
10
Epinephrine
Inulin
1 2 3 4
1 2 3 4


C. CORNEA
mcg Drug / g Tissue
7
6
5
4
3
2
1
Epinephrine
Inulin
1 2 3 4
1 2 3 4


D. IRIS - CILIARY BODY
mcg Drug / g Tissue
4
3
2
1
Epinephrine
Inulin
1 2 3 4
1 2 3 4


E. AQUEOUS HUMOR
mcg Drug / ml Fluid
0.20
0.15
0.10
0.05
Epinephrine
Inulin
1 2 3 4
1 2 3 4

and quenching, the data in counts per minute (cpm) due to tritiated epinephrine-HCl or inulin were converted to μg of drug per g of tissue or per ml of fluid through the use of standards, and normalized to amount of drug instilled. The counts due to [^{14}C]cholesterol were not converted to μg of cholesterol, however.

Throughout the study the surgical procedures were performed and completed within 5 min of sacrificing the rabbit so that errors due to redistribution of drug during the time required to obtain ocular tissue samples were minimized.

Results

Fig. 1 shows the concentration of epinephrine and inulin achieved in tears, conjunctiva, cornea, iris-ciliary body and aqueous humor at 30 min following instillation of various preparations of these compounds. Regardless of the preparation instilled, the concentration of epinephrine and inulin is the highest in tears, followed by the conjunctiva, cornea, iris-ciliary body and aqueous humor in that order. However, liposomal entrapment has the opposite effect of increasing the concentration of inulin in the conjunctiva, cornea and iris-ciliary body by 5–8-fold while decreasing that of epinephrine by 50%, indicating that liposomal entrapment of a drug may not necessarily yield the desired result of enhancing its absorption into the eye. In addition, Fig. 1 shows the vehicle effect liposomes can exert on the rank order of absorption efficiency of topically applied ophthalmic drugs. Consistent with the magnitude of their molecular weights, the lower molecular weight epinephrine is absorbed from an aqueous solution into the eye to a larger extent than the higher molecular weight inulin. Indeed, it is surprising to find that inulin can overcome the corneal barrier at all to gain entry to the internal eye despite its molecular size. However, this finding is consistent with that of Keller et al. (1980) who used [^{14}C]-labeled inulin in their study. It is equally surprising to find that the rank order just listed is reversed when both compounds are presented in liposomal form. Now more inulin than epinephrine is detected in the ocular tissues sampled, except for aqueous humor. A possible explanation for this finding is presented in the Discussion section.

Discussion

The manner by which liposomes alter the behavior of topically applied drugs in the eye cannot yet be predicted with certainty. Nevertheless, it is expected to be

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Fig. 1. Concentrations of epinephrine and inulin in (A) tears, (B) conjunctiva, (C) cornea, (D) iris-ciliary body, and (E) aqueous humor at 30 min following instillation of various preparations containing epinephrine or inulin. All concentrations were normalized to an instilled concentration of 0.5%. An average of 16 eyes was used for each preparation. Error bars, where shown, represent standard error of the mean. Key: 1 = free drug solution; 2 = mixture of free drug plus empty liposomes; 3 = free drug solution instilled 15 min after dosing with empty liposomes; 4 = liposome-entrapped drug.

influenced by 3 factors: affinity of the drug for the liposomes, alteration of membrane permeability by the phospholipids in liposomes, and structural integrity of the liposomes in tears, about the absorptive surfaces, and during and after transit across the cornea.

If a drug shows little affinity for the liposomes or if the liposomes lose their integrity in the tear pool as a result of interaction with tear proteins (Tall et al., 1978), liposomal entrapment of this drug would not be expected to improve its absorption characteristics when compared to its aqueous solution. From Fig. 1 it can be deduced that epinephrine effluxed readily from liposomes whereas inulin did not, an observation which was consistent with the fact that the efflux rate of compounds from liposomes can be affected by their water solubility characteristics (Juliano and Stamp, 1978). This was subsequently confirmed in experiments which evaluated the release of epinephrine and inulin from liposomes exposed to tear proteins (unpublished data). Consequently, the uptake of epinephrine by the ocular tissues/fluids sampled was not improved upon its encapsulation in liposomes. In fact, the concentration of epinephrine attained in the tear pool, the absorption site, was statistically the same ($P < 0.05$ by an F-test) whether epinephrine was entrapped in liposomes. Moreover, the rapid efflux of epinephrine from liposomes into the tear pool indirectly allowed its β-adrenergic action on the lacrimal gland (Diaz et al., 1980) to manifest, leading to an increase in tear production thus accelerating the removal of liposomes and drug alike from the tear chamber. It is for this reason that the radioactivity due to [^{14}C]cholesterol, our marker for liposomes, was 5–10 times lower in experiments involving epinephrine than in experiments involving inulin or no drug, i.e. empty liposomes (data not shown).

Since most of the inulin remained in association with liposomes, its clearance from the tear chamber would be controlled by that of liposomes. Fig. 1A shows that when presented in liposomal form the concentration of inulin in tears was 10 times higher when compared to its aqueous solution suggesting that liposome-encapsulated inulin disappeared at a slower rate from the tear pool. Unfortunately, the precise mechanism by which this prolonged retention of inulin in the tear pool was effected is unknown. However, it is unlikely to arise from physical blockage of the drainage apparatus by liposomes, in the light of the finding that neither the instillation of a mixture of free drug and empty liposomes nor predosing the rabbit eye with empty liposomes prior to solution instillation significantly increased the concentration of inulin (or epinephrine) in tears (Fig. 1A). Nonetheless, this elevated concentration of inulin in the tear pool may, in part, promote the uptake of inulin by the cornea and conjunctiva observed in the present study.

Another liposome-related factor that may lead to a higher concentration of inulin in the cornea and conjunctiva is alteration of membrane permeability by liposomes. According to prevailing theories (Kimelberg and Mayhew, 1978) this can be accomplished through either adsorption of liposomes to cell surfaces, endocytosis of liposomes by cells, or fusion of liposome bilayer with plasma membrane. However, it is not known which one of these possibilities applies to the absorption of liposome-encapsulated inulin. This is currently being investigated in our laboratory. For the time being, while [^{14}C]cholesterol was detected in the conjunctiva, cornea and

iris-ciliary body following topical instillation of liposomes, this cannot be construed as evidence for endocytosis or fusion, because cholesterol, like other phospholipid constituents, readily exchanges with its counterpart in cell membranes upon contact with the respective cell (Poznansky and Lange, 1978). On the basis of our present findings, a plausible explanation for the increased uptake of inulin by the cornea and conjunctiva is physical adsorption of liposomes onto conjunctival and corneal surfaces followed by partitioning of the entrapped drug into the milieu surrounding the liposomes with the simultaneous establishment of a high local concentration of inulin about the absorptive surfaces. This possibility was also invoked by Schaeffer and Krohn (1982) to account for the enhancement in corneal absorption of penicillin-G and indoxole by liposomes.

According to this mechanism, the eventual improvement in drug uptake by ocular tissus would depend largely on the relative affinity of the drug for the liposomes, the tear fluid and extraocular tissues like the cornea and conjunctiva. Thus, if a drug readily partitions out of the liposome into the tear fluid, which seems to be the case for epinephrine, there may not be any improvement in its uptake by liposomes. In this case, the extent of uptake would be comparable to that achieved from an aqueous solution of the drug at best. Interestingly, the concentration of epinephrine in the ocular tissues (fluids) assayed following liposomal entrapment were actually lower than that achieved from aqueous solutions of the drug (Fig. 1). This suggests that the phospholipids in the liposomes may have partially restored the structural integrity and therefore barrier properties of the corneal and conjunctival epithelia, which were breached as a result of their exposure to the acidic solution instilled (pH = 4) (Keller et al., 1980). This speculation is supported by the parallel findings that the concentration of epinephrine achieved in the conjunctiva, cornea, iris-ciliary body and aqueous humor was also reduced when administered either in combination with empty liposomes or 15 min following instillation of empty liposomes (Fig. 1).

In the case of inulin, this possible negative effect of phospholipids on the permeability properties of the cornea and conjunctiva was more than offset by the high local concentration of inulin attained near the absorptive surfaces, as proposed earlier. As is the case with penicillin-G (Schaeffer and Krohn, 1982), liposomal entrapment of inulin is requisite to the 5–8-fold enhancement in permeation, which was not observed when inulin was administered in conjunction with empty liposomes (Fig. 1). Oddly enough, no inulin was detected in the aqueous humor when presented in liposomal form, despite its higher concentration in the cornea and iris-ciliary body, the latter tissue deriving their inulin from the aqueous humor. There is no forthcoming explanation for this finding at this time.

In summary, given these preliminary data, it would be premature to prognosticate the therapeutic usefulness of liposomes in topical ocular drug delivery. There is circumstantial evidence, however, that through its influence on the local concentration of drug established at the absorbing surface, the affinity of a drug for the liposome is a significant factor in determining whether liposomal entrapment would enhance its corneal absorption. Work is in progress to investigate the role of molecular size and lipophilic characteristics of drugs in affecting their kinetics of disposition in the tear film and intraocular tissues following liposomal entrapment.

and to elucidate the interaction of liposomes with the component layers of the cornea.

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