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Tear-induced release of liposome-entrapped agents

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Summary

The potential use of liposomes for the ocular delivery of entrapped substances appears promising, but very little is known about the possible effect of tear fluid on the stability of liposomal preparations. In this study, we have continued our investigations of tear-mediated release of liposome-encapsulated agents with the aim of developing a biocompatible carrier for topical ocular applications. Rabbit tear fluid was shown to promote the release of both an entrapped water-soluble dye (5-carboxyfluorescein) and a high molecular weight protein (acetylcholinesterase) from multilamellar liposomes. Heat treatment of tear fluid was found to have no effect on the release of the enzyme but did reduce dye leakage. Pre-incubation of the tear fluid with empty liposomes was found to have no significant effect on either enzyme or dye release. Unlike serum where liposome destabilization is reportedly almost entirely the result of high density lipoproteins, our results suggest that multiple factors are involved in tear-mediated liposome destabilization.

Introduction

The successful application of liposomes as delivery devices for drugs and other bio-active compounds in vivo is dependent on the stability of the carrier in the biological milieu. The fate of liposomes in the blood stream is perhaps the best understood and their interactions with serum proteins have been recently reviewed by Bonte and Juliano (1986). The interactions are complex and include lipid exchange, protein binding with possible changes in cellular uptake and vesicle aggregation, as well as vesicle disruption. The limited success that has been achieved in the use of liposomes for systemic delivery is largely the result of their interactions with serum components, uptake by cells of the reticuloendothelial system (Roerdink et al., 1984), and the inability of liposomes to move into extravascular spaces (Poste et al., 1982).

The use of liposomes as drug carriers has shown some success when they are delivered directly where required. For example, intravitreal injection of drug-containing liposomes has demonstrated an improved therapeutic response over the free drug (Tremblay et al., 1985; Fishman et al., 1986). As well, ocular drug delivery utilizing liposomes in a topical formulation has been shown to be advantageous in several animal studies (Smolin et al., 1981; Schaeffer and Krohn, 1982; Singh and Mezei, 1983; Lee et al., 1985; Norley et al., 1986). If liposomes can increase the residency time of

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encapsulated agents in the pre-corneal area, they may serve as suitable carriers for prophylactic agents which could protect the eye against chemical insults. Indeed, we have shown that multilamellar liposomes containing acetylcholinesterase can provide significant protection against chemically induced miosis in rabbits for several hours after instillation (Shek and Barber, 1987). All of these studies indicate that liposomes can alter the bioavailability of encapsulated drugs or biological agents and may provide sustained drug levels which cannot be obtained using conventional eye drops or ointments.

Any therapeutic agent applied topically to the eye comes into immediate contact with tear fluid. If a delivery vehicle is employed for the controlled release of an entrapped agent, it is important to determine the potential impact of tear fluid on the stability of the vehicle for the optimal design of specific formulations. Although a variety of liposomal formulations have been used for the ocular delivery of encapsulated substances, very little information is available on potential interactions between tear fluid and liposomes (Stratford et al., 1983a,b). In a previous report, we demonstrated that tear fluid can indeed destabilize liposomes in a manner very similar to that observed to occur in the presence of serum (Barber and Shek, 1986). Rabbit tear fluid induces rapid release of the water-soluble dye, carboxyfluorescein, from unilamellar liposomes. The release of this dye can be modulated by alterations in the cholesterol content of the liposomal bilayer. In this study, we demonstrate that multilamellar vesicles are also susceptible to tear-mediated dye release and that tear fluid can also induce the release of acetylcholinesterase, a high molecular weight protein. An attempt to characterize the factor(s) involved in tear-mediated leakage by prior heat-treatment and incubation with empty liposomes is described.

Materials and Methods

Animals and tear collection. The animals used were normal female New Zealand White rabbits, 12–14 weeks old and 2.0–2.5 kg in weight. Tears were collected from the lower eye-lid margins of rabbits with 10-μl micropipettes. Extreme care was taken to avoid irritating the eye so as not to stimulate tearing. Animal care and treatment in this investigation were in accordance with the principles contained in the Guide to the Care and Use of Experimental Animals as prepared by the Canadian Council on Animal Care and with the ARVO Resolution on the Use of Animals in Research.

Chemicals. Egg phosphatidylcholine, cholesterol, stearylamine, acetylcholinesterase (type V-S), and Hepes were purchased from Sigma (St. Louis, MO). [1,3-^{3}H]Diisopropylfluorophosphate (3.0 Ci/mmol) and [4-^{14}C]cholesterol (57.5 mCi/mmol) were purchased from New England Nuclear (Boston, MA). 5-Carboxyfluorescein was purchased from Calbiochem-Behring (La Jolla, CA). All other chemicals were reagent grade.

Radiolabelling of acetylcholinesterase. The active site of the enzyme was labelled using [^{3}H]DFP as described by Tiller and Struve (1985). After dialysis, protein concentration was determined using the Bio-Rad protein assay (BioRad, Richmond, CA). The average specific activity obtained was 2.05 μCi/mg protein.

Preparation of liposomes. Freeze-dried multilamellar liposomes were prepared essentially as described by Kirby and Gregoriadis (1984). Egg phosphatidylcholine, cholesterol, and stearylamine were combined in a molar ratio of 7:2:1 in organic solvent and dried under a stream of nitrogen; a total of 66 μmol lipid were used. For acetylcholinesterase release experiments, a trace amount of [^{14}C]cholesterol was also included. Residual solvent was removed in vacuo for a minimum of 30 min.

For experiments in which carboxyfluorescein was entrapped, the dried lipid film was rehydrated with 1 ml of 100 mM carboxyfluorescein, 50 mM Hepes (pH 7.4) by vigorous vortexing. The liposomes were sonicated under nitrogen at 100 W for 3 min using a probe sonicator (Braunsonic 1510). The preparation was then split into two equal aliquots and lyophilized overnight. For experiments in which acetylcholinesterase was entrapped, multilamellar liposomes were first formed by hydration with 0.5 ml of distilled water. These

were then sonicated as described above after which a total of 2 mg of enzyme was added in 2 ml of 10 mM Na_2HPO_4, 0.1 M NaCl (pH 7.0). This usually included approx. 1 mg of radiolabelled enzyme. The preparation was then split into aliquots of 0.8 ml and freeze-dried.

Carboxyfluorescein release assay. For each experiment, one freeze-dried aliquot was rehydrated with 200 μl of distilled water for 1 h at room temperature under nitrogen. The re-formed vesicles were then washed and tear-induced dye release was monitored as previously described (Barber and Shek, 1986) after combining equal volumes of washed liposomes with buffer, normal rabbit tears, or pre-treated rabbit tears. All samples were incubated at 37°C during the release studies. For each sample, the percent remaining carboxyfluorescein (latency) was calculated using the values obtained in the presence of detergent as 100% efflux. Two pre-treatments were used and included heat inactivation at 95°C for 30 min or pre-incubation with empty liposomes of the same lipid composition for 1 h at 37°C (45 μl of tears were combined with 0.66 μmol total lipid). The initial half-times for carboxyfluorescein leakage were calculated according to Allen and Cleland (1980) using the initial linear portion of the plots of log[carboxyfluorescein latency] vs time. Long-term half-times were similarly calculated and are based on release kinetics after 1 h.

Acetylcholinesterase release assay. For each experiment, one aliquot of freeze-dried sample was rehydrated overnight with 33 μl of distilled water at 4°C under nitrogen. The re-formed liposomes were then washed three times in 8 ml of 10 mM Na_2HPO_4, 0.1 M NaCl (pH 7.0) buffer by pelletting at 110000 × *g* for 20 min at 5°C. The final pellet was gently resuspended with 550 μl of buffer. Aliquots (150 μl) of the liposomes were incubated with an equal volume of either buffer, rabbit tears, or pre-treated rabbit tears. The three pre-treatments included heat-treatment at either 56 or 95°C for 30 min and pre-incubation as before with empty liposomes (2 μmol liposomal lipid mixed with 150 μl tears) having the same lipid composition.

The samples were made 20% (w/v) with solid sucrose and layered over 0.4 ml of 40% CsCl. A discontinuous gradient was constructed over the sample by layering 3.7 ml of 10% sucrose in buffer followed by 0.6 ml of buffer. The samples were then centrifuged at 225000 × *g* at 5°C in a Beckman SW50.1 rotor for 2.5 h. The tubes were fractionated from the bottom (fraction size: 0.3 ml) and each fraction was counted in a Beckman LS5801 scintillation counter. Beckman Ultra-Clear centrifuge tubes were pre-coated with polyvinyl alcohol to make them hydrophilic (Holmquist, 1982).

Ellman assay. The proportion of enzyme released in the presence of tear fluid was also determined using the method of Ellman et al. (1961) in both the presence and absence of detergent according to Hall and Brodbeck (1978). Liposomes were incubated at 37°C with an equal volume of either tears or buffer for 3 h. Aliquots were removed at intervals for enzymatic assay. The assay mixture contained 0.1 ml of 30 mM acetylthiocholine, 3.75 mM 5,5-dithionitrobenzoic acid in 0.1 M Na_2HPO_4 (pH 7.4), plus 2.9 ml of 10 mM Tris-HCl, 0.1 M NaCl (pH 7.4). Total activity was determined in the presence of 1% (v/v) Triton X-100. The change in absorbance was followed at 412 nm. Latency was calculated as the proportion of activity released in the presence of detergent.

Results

Tear-induced release of acetylcholinesterase

A discontinuous sucrose density gradient was used to separate liposomes and associated acetylcholinesterase from free enzyme. Typical results obtained using this flotation procedure are shown in Fig. 1 where it can be seen that the ^{14}C-labelled liposomes migrate to the top phase. Practically all of the ^{14}C label could be recovered at the top interface. Unassociated (free) enzyme remained at the bottom of the tube and could be collected in the first four fractions while liposome-associated acetylcholinesterase was cleanly separated and was collected in the top five fractions (Fig. 1). When the free enzyme alone was centrifuged in this gradient, all of the activity remained in the bottom four fractions (data not shown). The

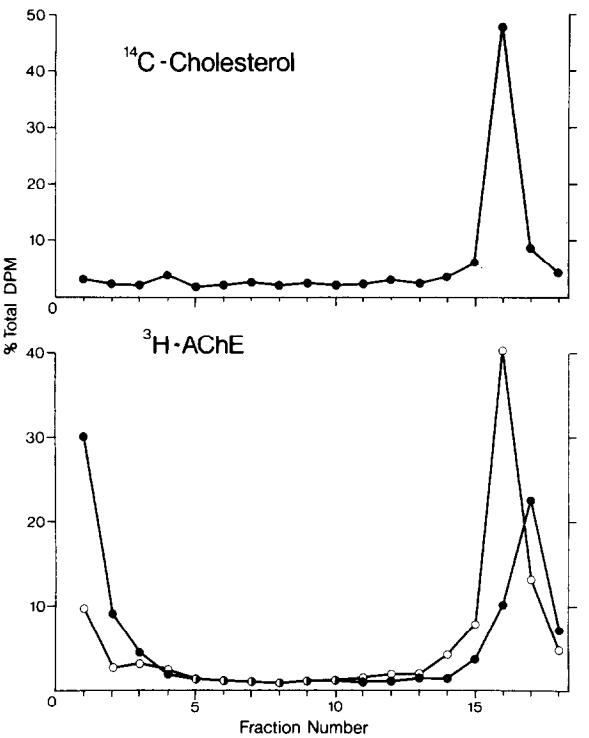

Fig. 1. Distribution of [^{14}C]cholesterol and [^{3}H]acetylcholinesterase after density gradient centrifugation. Liposomes were incubated with an equal volume of buffer (○) or tear fluid (●). The gradient was constructed as described in Materials and Methods; the top of the gradient is at the right.

incorporation efficiency for acetylcholinesterase was determined by this method to be 73.3 ± 0.9%.

For the release experiments, liposomes and tears were combined as described in Materials and Methods and one aliquot was immediately placed on ice and the sucrose gradient was prepared as quickly as possible and centrifugation begun. Usually this required approx. 15 min which we have reported as our minimum incubation time. In the absence of tear fluid, 25.6% of the associated enzyme was released from the vesicles with no change after a 3 h incubation at 37 °C. Incubation with untreated rabbit tear fluid resulted in 42.8% release at 15 min with essentially no increase after 3 h (Table 1).

Tear-mediated enzyme release was not significantly reduced following dilution of tear fluid to either 50 or 25% with buffer. Dilution to 10% did, however, result in a significant reduction in tear-mediated release (Fig. 2).

TABLE 1

The effect of tears on the release of liposome-associated [^{3}H]acetylcholinesterase as determined by flotation analysis

Liposomes were incubated with an equal volume of tear fluid for the indicated times before flotation on discontinuous sucrose density gradients as described in Materials and Methods. Samples which were incubated for 180 min were kept at 37 °C. Tears were heat-treated by incubation at either 56 or 95 °C for 0.5 h prior to use. Data represent means ± S.E. from at least three independent experiments.

Incubation time (min)	Enzyme release (%)			
	Buffer	Normal tears	Heat-inactivated (56 °C)	Heat-inactivated (95 °C)
15	25.6 ± 2.0	42.8 ± 2.0	42.1 ± 1.1	45.0 ± 3.6
180	27.7 ± 0.8	46.7 ± 3.9	46.8 ± 3.1	51.6 ± 4.3

Using the Ellman assay to measure enzyme latency, a similar tear-induced release of AChE

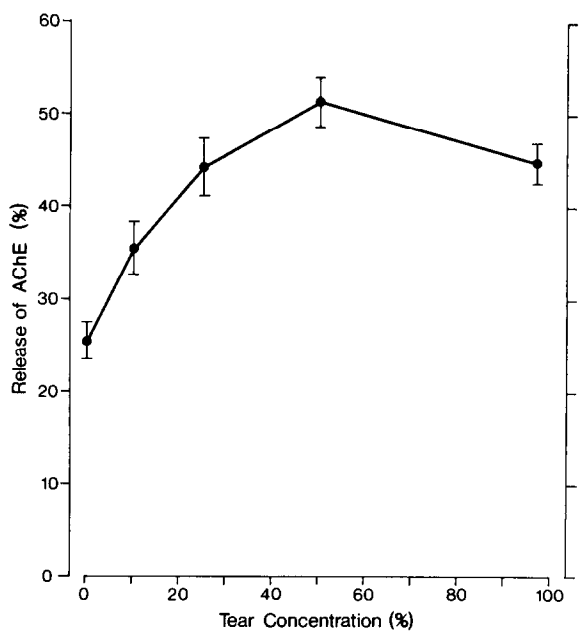

Fig. 2. Effect of rabbit tear dilution on the release of liposome-associated acetylcholinesterase. Liposomes were combined with an equal volume of tears, or tears diluted with buffer, and then immediately subjected to density gradient centrifugation as described. Data represent means ± S.E. from at least three independent experiments.

TABLE 2

The effect of tears on the release of liposome-associated acetylcholinesterase as determined by enzyme latency

Liposomes were incubated with an equal volume of tear fluid and aliquots were assayed for acetylcholinesterase activity as described in Materials and Methods at the indicated times. Data represent means ± S.E. from three independent experiments.

Incubation time (min)	Enzyme latency (%)	
	Buffer	Normal tears
0	86.9 ± 0.9	86.9 ± 0.9
7	86.3 ± 1.9	68.0 ± 3.3
15	84.6 ± 0.9	68.5 ± 4.9
30	84.4 ± 1.5	65.1 ± 3.4
60	84.8 ± 2.5	63.0 ± 3.8
240	82.5 ± 1.2	62.7 ± 3.0

was observed (Table 2). In the presence of buffer only, enzyme latency declined from 86.9 to 82.5% over the 3 h incubation period. When incubated with an equal volume of tear fluid, enzyme latency dropped to 68.0% after 7 min and to 62.7% at the end of the 3 h incubation. Based on the initial latency, the proportion of liposome-associated AChE exposed on the outer bilayers was 13.1% ± 0.8.

The effect of tear pre-treatment on acetylcholinesterase release

Heat treatment at two different temperatures was used in an attempt to inactivate the tear factors involved in enzyme release. Tear fluid was heated to either 56 or 95 °C for 30 min prior to the addition of liposomes containing acetylcholinesterase. Flotation revealed no effect of either of these treatments on the extent of acetylcholinesterase release relative to normal untreated tear fluid after incubation for either 15 min or 3 h (Table 1).

The other pre-treatment of tear fluid consisted of pre-incubation with empty multilamellar liposomes of the same lipid composition as those used for enzyme entrapment. At the end of this pre-incubation at 37 °C for 1 h, there was no obvious change in the turbidity of the tear/liposome mixture. Furthermore, the activity of the preincubated tear fluid was not found to be significantly different from that of untreated tears in inducing the release of liposome-entrapped acetylcholinesterase. The extent of release in each case was about 46%.

The effect of tear pre-treatment on carboxyfluorescein release

Liposomal carboxyfluorescein latency as a function of time was determined in the presence of buffer, tears, and pre-treated tears. These leakage experiments revealed that incubation with untreated tears resulted in a rapid release of dye from multilamellar liposomes (Fig. 3). The initial latency was measured to be 58.9% and dropped quickly to 44.3% by 30 min and then decreased only slightly over the remaining 2 h. This compares to an initial entrapment of 71.5% in controls with a slight decrease to 68.5% over the same period. Incubation with tears which had been pre-incubated with empty liposomes resulted in no reduction in the extent of dye release. The initial latency measured in all of these experiments should have been the same at time zero, since the same

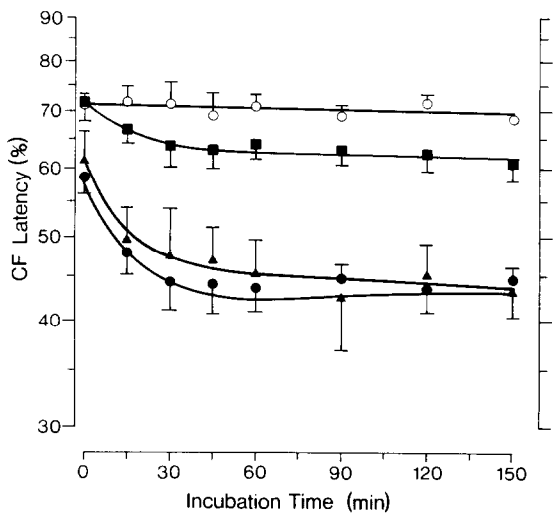

Fig. 3. Effect of tear pre-treatment on liposomal carboxyfluorescein (CF) latency. Dye-containing liposomes were incubated at 37 °C for various time periods with an equal volume of buffer (○), untreated tears (●), heat-treated (95 °C for 30 min) tears (■), or tears pre-incubated with empty liposomes (▲). Data represent means ± S.E. from at least three independent experiments.

TABLE 3

Half-times for carboxyfluorescein release from liposomes in the presence of rabbit tears, heat-treated tears (95 °C for 30 min), and tears pre-incubated with empty liposomes

The determination of the two half-times is described in Materials and Methods. Data represent means ± S.E. from at least three independent experiments.

	Half-times for carboxyfluorescein release (h)	
	Initial release	Long-term release
Buffer	30.75 ± 0.68	30.75 ± 0.68
Normal tears	0.85 ± 0.08	25.77 ± 2.40
Heat-treated tears	5.12 ± 0.76	27.97 ± 7.36
Pre-incubated tears	1.41 ± 0.36	27.31 ± 2.95

liposome preparation was used in each set of experiments. The 10% difference in initial latencies for liposomes incubated with either untreated or pre-incubated tears indicates a very rapid release of dye occurring within seconds of mixing with tear fluid. Tears which had previously been heat treated at 95 °C for 30 min induced considerably less dye release. Carboxyfluorescein latency was initially measured to be the same as controls and declined to 63.8% after 30 min with a further gradual drop to 61.0% at 2.5 h.

The half-times for dye release were calculated from the semi-logarithmic plots and are shown in Table 3. Incubation with tear fluid significantly reduced the half-times measured during the initial 30 min of incubation. The half-times decreased from 30.75 h for liposomes incubated in buffer to 0.85 h using untreated tear fluid. Heat-treatment and pre-incubation of the tear fluid reduced the rate of initial dye release (relative to untreated tears) with observed half-times of 1.41 and 5.12 h, respectively. There was no significant difference in the long-term half-times among any of the treatments.

Discussion

We have previously reported that rabbit tear fluid induces a rapid and substantial leakage of carboxyfluorescein from unilamellar liposomes prepared from egg phosphatidylcholine and stearylamine in a 9:1 molar ratio (Barber and Shek, 1986). Furthermore, we demonstrated that both the extent and the rate of dye release could be reduced substantially by the incorporation of cholesterol into the liposomal bilayers. A similar destabilization of liposomal integrity has been well documented to occur in the presence of serum (Scherphof et al., 1978; Finkelstein and Weissmann, 1979) as has the ability of cholesterol to prevent or at least reduce serum-induced leakage (Finkelstein and Weissman, 1979; Allen, 1981).

The liposomes used in this study were multilamellar vesicles prepared by a freeze-drying method. This method provides higher entrapment efficiencies than most other methods as the liposomes re-form during rehydration in the presence of high solute concentrations (Kirby and Gregoriadis, 1984). Furthermore, it avoids exposure of labile solutes (e.g., enzymes) to organic solvents, sonication, or other harsh conditions used in some other preparation methods. The lipid composition used was egg phosphatidylcholine, cholesterol, and stearylamine in a 7:2:1 molar ratio. This lipid composition was selected as it was efficacious for in vivo prophylaxis (Shek and Barber, 1987). Unilamellar liposomes of this composition containing carboxyfluorescein are clearly susceptible to tear-induced dye release (Barber and Shek, 1986). The multilamellar liposomes were similarly shown to be destabilized by tear fluid with near maximal dye release occurring within 30 min (Fig. 3).

The two liposome-entrapped substances used in this study have different physical and biochemical properties. Carboxyfluorescein is a low molecular weight water-soluble dye commonly used as a model solute to study liposome permeability. It is presumed not to interact with lipid bilayers. The species of acetylcholinesterase used in this study have molecular masses varying from 4×10^5 to 1.15×10^6 Da (Massoulie and Bon, 1982) and are also water-soluble. They have been reported to associate with phospholipid bilayers, most likely through ionic interactions (Watkins et al., 1977; Kaufman and Silman, 1980; Cohen and Barenholz, 1984; Tiller and Struve, 1985).

Exposure to tear fluid caused a very rapid maximal release of associated enzyme. Similar re-

sults were obtained by both the flotation and latency methods employed in this study. Relative to controls, 17% more enzyme was released after 15 min with no further significant release following a 3 h incubation at 37 °C (Table 1). Dilution of the tear fluid had no effect until concentrations of less than 25% were employed (Fig. 2). Based on the latency experiments, enzyme exposed on the outer surface amounted to 13.0% of the total liposome-associated AChE. This enzyme was likely released during the flotation experiments as a result of the high salt (40% CsCl) cushion used (Cohen and Barenholz, 1984; Tiller and Struve, 1985) and contributed to the 25.6% release observed in the buffer-incubated controls. The remainder of the released enzyme must have been released from the liposomal interior, perhaps due to osmotic forces.

The release of entrapped solutes observed in the presence of serum can be attenuated by prior heat treatment at 56 °C or pre-incubation with phospholipid vesicles. Heat treatment at 56 °C is commonly used to denature components of the complement system which has been shown to participate in mediating liposomal lysis (Hesketh et al., 1971; Shin et al., 1978). Other studies investigating the involvement of high density lipoproteins in liposome destabilization have reported both susceptibility to heat-treatment (Senior et al., 1983; Tall et al., 1983; Senior and Gregoriadis, 1984) and resistance (Allen and Cleland, 1980; Kirby et al., 1980).

The results of our studies with acetylcholinesterase indicate that there was no effect on tear-mediated release when tears were heat-treated at either 56 or 95 °C for 30 min prior to incubation with enzyme-containing liposomes (Table 1). However, the leakage of carboxyfluorescein was reduced by prior heat-treatment at 95 °C (Fig. 3) and the initial half-time for dye release was increased, but still significantly less than that of buffer-incubated controls (Table 3). Following the initial rapid release which occurs during the first 30 min, the rate of leakage was essentially the same as that observed in the control experiments.

Uptake of liposomal phospholipid by high density lipoproteins, with consequent vesicle leakage, can be pre-saturated by incubation with susceptible liposomes (Lelkes and Tandeter, 1982). Pre-incubation of tear fluid with liposomes in this study, however, revealed no significant reduction in the subsequent release of either carboxyfluorescein (Fig. 3) or acetylcholinesterase. The release of both carboxyfluorescein and acetylcholinesterase was similar in that it occurred during approximately the same time-span and to approximately the same extent. The failure to observe any further release of enzyme after 15 min and the similarity in the long-term half-times for dye release suggest that the initial leakage was the result of limited liposome lysis induced by tear fluid. This lytic effect could not be reduced by prior incubation with phospholipid.

The failure of heat treatment to reduce the extent of enzyme release while similar treatment significantly reduced release of the much smaller carboxyfluorescein molecule is difficult to reconcile. The release of such large protein molecules is difficult to imagine without actual lysis of liposomal lamellae but, were this to occur, dye molecules would also be released. Acetylcholinesterase is reported to associate with bilayers to some extent and perhaps this renders the membrane susceptible to some heat-stable factors present in tears. For example, antibodies are heat-stable and could bind to liposome-associated enzyme if antibodies to it existed. However, the lysis that follows antibody binding is dependent on the complement cascade which is easily denatured by heat-treatment. Thus, our observation renders it unlikely that the release mechanism is a complement-dependent and antibody-mediated event, although immunoglobulins are present in tear fluid (Allansmith, 1973).

In summary, rabbit tear fluid can induce a significant release of liposome-entrapped agents of low and high molecular weights, as exemplified by carboxyfluorescein and acetylcholinesterase, respectively. Pre-treatment of tears by heat or by pre-incubation with liposomal lipids resulted in different effects on the subsequent release of entrapped agents. Therefore, the results of this study suggest that multifactorial mechanisms might be operating in tear-induced liposome destabilization. The physicochemical properties of the active tear factor(s) remain to be established.

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