THE JOURNAL OF BIOLOGICAL CHEMISTRY

Vol. 269, No. 10, Issue of March 11, pp. 7364-7370, 1994
Printed in U.S.A.

Influence of Plasma on the Osmotic Sensitivity of Large Unilamellar Vesicles Prepared by Extrusion*

(Received for publication, June 11, 1993, and in revised form, November 16, 1993)

Barbara L.-S. Mui‡, Pieter R. Cullis‡, P. Haydn Pritchard§, and Thomas D. Madden¶‖

From the Departments of ‡Biochemistry, §Pathology, and ¶Pharmacology and Therapeutics, University of British Columbia, Vancouver, British Columbia V6T 1Z3, Canada

In the presence of plasma, the osmotic differential required to trigger lysis of large unilamellar vesicles is significantly decreased with the membrane tension at rupture being reduced from about 36 to about 12 dynes/cm for vesicles composed of palmitoyloleoylphosphatidylcholine: cholesterol (55:45). Despite increasing vesicle sensitivity, however, plasma does not alter the characteristics of osmotically induced lysis. As in the absence of plasma, lysis is not an all-or-nothing event but instead results in only partial loss of intravesicular solute, so that following membrane resealing the vesicle interior remains hyperosmotic with respect to the external medium. To identify the component responsible for the observed increase in vesicle osmotic sensitivity, plasma was fractionated by density centrifugation. Albumin and other soluble plasma proteins, including those associated with the complement system, were found to exert only a modest influence on vesicle osmotic behavior. In contrast all of the lipoprotein fractions lowered vesicle tolerance to osmotic pressure, with high density lipoprotein exerting an effect comparable to whole plasma.

A number of earlier studies have examined the influence of plasma or serum on the physical properties of liposomes; this research was stimulated in part by the therapeutic potential of liposomal drug delivery systems. The incorporation of cholesterol into SUVs[1] composed of phosphatidylcholine was shown to stabilize these vesicles (Kirby *et al.*, 1980) preventing breakdown due to assimilation of liposomal phospholipid into high density lipoproteins (Scherphof *et al.*, 1978; Scherphof and Morselt, 1984). However, even liposomal systems that appear physically stable in plasma or serum often exhibit increased rates of solute leakage (Allen and Cleland, 1980; Comiskey and Heath, 1990), probably as the result of protein interactions with the liposome membrane (Weinstein *et al.*, 1981; Allen *et al.*, 1985). Several lines of evidence suggest that these interactions involve penetration of the lipid bilayer by a hydrophobic or amphipathic protein domain. Serum-induced leakage, for example, is less pronounced for liposomal systems composed of cholesterol with either saturated phosphatidylcholines or sphingomyelin (Gregoriadis and Senior, 1980; Damen *et al.*, 1981; Allen and Everest, 1983). This would be expected if protein insertion were sensitive to the lipid packing density. Second, the presence of bilayer defects, such as those arising at the phase boundaries between gel and liquid-crystalline domains, greatly favors protein insertion and solute release (Pownall *et al.*, 1979; Kamellis *et al.*, 1980; Klausner *et al.*, 1985; Epand *et al.*, 1989). Furthermore, liposomal systems containing high molar ratios of negatively charged phospholipids, such as phosphatidylglycerol or phosphatidylinositol, show greater serum-induced leakage compared to equivalent neutral systems. This effect, which can be abolished by increasing the medium ionic strength, suggests that charge repulsion between adjacent phospholipids may reduce packing densities, thereby facilitating protein penetration (Cominsky and Heath, 1990).

While the studies cited above have examined the structural stability and bilayer permeability of liposomes in the presence of serum or plasma, little attention has been focused on how plasma proteins may influence the osmotic stability of liposomes. The only published research in this area consists of experiments briefly reported by Allen and Cleland (1980) and Allen *et al.* (1992), indicating that serum-induced leakage is increased for vesicles exposed to an osmotic gradient. We have recently characterized the osmotic properties of LUVs prepared using the extrusion technique (Mui *et al.*, 1993). When such vesicles are placed in a solution that is hypoosmotic with respect to the intravesicular medium, the resulting influx of water first causes the vesicles to assume a spherical shape and can then create an osmotic pressure. This pressure results in an elastic expansion of the lipid bilayer (Evans and Needham, 1987; Needham and Nunn, 1990) and for osmotic differentials of sufficient magnitude can produce membrane rupture. We have shown that lysis results in only partial release of intravesicular solute such that the intravesicular medium remains hyperosmotic following bilayer resealing (Mui *et al.*, 1993). In the present report, we have extended this work to characterize the influence of plasma on the osmotic behavior of LUVs and have also identified the plasma component responsible for the observed increase in vesicle osmotic sensitivity.

MATERIALS AND METHODS

1-Palmitoyl- 2-oleoylphosphatidylcholine (POPC), and monooleoyl phosphatidylcholine were obtained from Avanti Polar Lipids (Alabaster, AL). Cholesterol (standard for chromatography), oleic acid, and fatty acid-depleted bovine serum albumin and human serum albumin were purchased from Sigma. [^{14}C]Citrate, [^{3}H]dextran (average M_r 70,000), [^{3}H]cholesteryl hexadecylether, and [^{3}H]glucose were from DuPont NEN, while [^{14}C]dipalmitoylphosphatidylcholine was obtained from Amersham Corp. 5(6)-Carboxyfluorescein was purchased from Eastman Kodak and purified according to Weinstein *et al.* (1984). Rabbit anti-sheep polyclonal antibody was purchased from Cedar Lane (Ontario, Canada).

Preparation of Large Unilamellar Vesicles—Lipid mixtures were prepared by colyophilization from benzene:methanol (95:5, v/v) under high vacuum (<0.1 millitorr) for a minimum of 4 h, protected from light. Unless otherwise stated mixtures of palmitoyloleoylphosphatidylcholine and cholesterol were prepared in a 55:45 molar ratio. Large

* The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‖ To whom correspondence should be addressed.

[1] The abbreviations used are: SUV, small unilamellar vesicle; POPC, 1-palmitoyl-2-oleoylphosphatidylcholine; LUV, large unilamellar vesicle; DGVB, dextrose:gelatin:Veronal-buffered saline; SRB, sheep red blood cell; VLDL, very low density lipoprotein; LDL, low density lipoprotein; HDL, high density lipoprotein.

multilamellar vesicles were prepared by hydrating dry lipid in the appropriate solution, and the dispersion was then freeze-thawed five times employing liquid nitrogen to promote equilibrium transmembrane solute distribution (Mayer *et al.*, 1985). Large unilamellar vesicles were then prepared by extruding the frozen and thawed large multilamellar vesicles 10 times through two stacked 100-nm pore size filters (Nuclepore) using an Extruder (Lipex Biomembranes, Inc., Vancouver, Canada) as previously described (Hope *et al.*, 1985).

Blood Collection—Blood was collected from a normal, fasting human volunteer in EDTA or silicone-coated tubes for the isolation of plasma or serum, respectively. The tubes were spun at 2000 × *g* to pellet the red cells. Sodium azide (0.03%) was included in the plasma prior to the lipoprotein fractionation protocol.

Vesicle Trapped Volume Measurements—Large unilamellar vesicles prepared by extrusion are not spherical and, when placed in a hypoosmotic solution, will initially "round up" in order to maximize their volume to surface area ratio (Mui *et al.*, 1993). The increase in trapped volume associated with this morphological change was determined for LUVs composed of POPC:cholesterol. Lipid labeled with 9×10^{-4} μCi of [^{14}C]dipalmitoylphosphatidylcholine/μmol of phospholipid was hydrated in 10 mM NaCl, 1 mM glucose (1.5 μCi ml^{-1} [^{3}H]glucose), freeze-thawed five times as described above, and extruded at 45 °C through 100-nm pore size filters. These vesicles were then diluted 5 fold into either 1 mM glucose (1.5 μCi ml^{-1} [^{3}H]glucose) or 10 mM NaCl, 1 mM glucose (1.5 μCi ml^{-1} [^{3}H]glucose). The samples were filter-sterilized using 0.22-μm polycarbonate filters (Nuclepore Corp.) and incubated at 45 °C. At various times, trapped volumes were determined by passing aliquots (500 μl) of the vesicle suspension down 9-ml pre-packed Sephadex G-25 columns (Pharmacia LKB Biotechnology Inc.) pre-equilibrated with 10 mM citrate, 5 mM HEPES pH 7.4 to remove external [^{3}H]glucose.

Determination of Osmotically Induced Solute Release from LUVs—Vesicles (50 mg ml^{-1} total lipid) were prepared in 700 mM NaCl, 100 mM carboxyfluorescein, 20 mM HEPES pH 7.4 (1700 mosm/kg). To remove unencapsulated carboxyfluorescein, an aliquot (100 μl) was passed down a Sephadex G-50 (medium) column (1.5 × 10 cm) pre-equilibrated with an isoosmotic solution (850 mM NaCl, 20 mM HEPES pH 7.4) and the peak lipid fraction collected. Carboxyfluorescein is a convenient marker for solute release; at the high intravesicular concentrations employed, fluorophore quenching is essentially complete; only following leakage and consequent dilution in the external medium can a fluorescent signal be detected (for review, see Weinstein *et al.* (1984)). To determine the kinetics and extent of osmotically induced lysis, LUVs were diluted 1:100 (final lipid concentration 60 μM) into buffered glucose-NaCl solutions containing the indicated concentrations of human serum albumin, plasma, bovine serum albumin, or lipoprotein fractions and incubated at 22 °C. Solution osmolarities were adjusted using glucose (0–1.4 M) with the NaCl concentration maintained at 150 mM. At various times, a 50-μl aliquot was diluted into 3 ml of the same osmolarity glucose-NaCl buffer and carboxyfluorescein fluorescence determined. This dilution was performed to minimize protein-mediated quenching (Lelkes and Tandeter, 1982). Total fluorophore release was achieved by the addition of octyl glucopyranoside (final concentration 25 mM) to the sample. Carboxyfluorescein fluorescence was measured using a Perkin-Elmer LS 50 spectrofluorometer at 492 nm (bandwidth 2.5 nm) excitation and 520 nm (bandwidth 5 nm) emission.

Influence of Solute Molecular Weight on Osmotically Induced Leakage—Vesicles of POPC:cholesterol (60 mg ml^{-1} lipid) were prepared in 850 mM NaCl, 20 mM HEPES pH 7.4 containing 4.4 μCi [^{14}C]citrate and 10 μCi ml^{-1} [^{3}H]dextran (average M_r 70,000). Aliquots were then diluted 1:40 with either 850 mM NaCl or 150 mM NaCl in the presence or absence of 10% plasma. After a 2-min incubation, the vesicles were passed down a Bio-Gel A-1.5m column (1.5 × 20 cm) pre-equilibrated with either 850 mM NaCl, 20 mM HEPES pH 7.4 or 150 mM NaCl, 20 mM HEPES pH 7.4. Retention of [^{14}C]citrate and [^{3}H]dextran was determined by liquid scintillation counting using a dual radiolabel program. Phospholipid concentrations were quantitated by phosphate analysis (see below).

Hemolytic Assay of Serum for Complement Activity—The hemolytic assay for complement activity was performed as described by Whaley (1985). Sheep red blood cells (SRBs) were incubated with rabbit anti-SRB polyclonal antibodies in DGVB (5 mM sodium barbital, pH 7.4, 75 mM NaCl, 2.5% glucose, 0.5 mM $MgCl_2$, 0.15 mM $CaCl_2$ and 0.1% gelatin) at 50 °C for 30 min. The antibody-coated cells were then washed three times with EGTA-DGVB (DGVB containing 40 mM EGTA) by pelleting (2000 × *g* for 5 min) and resuspension of the cells. The cells were then resuspended in ice-cold DGVB and kept on ice. Aliquots (100 μl) of the cells were then added 1:1 to DGVB solutions containing 0–100% serum and incubated at 37 °C for 30 min. If all components in the classical complement cascade (which shares components in the last steps of the alternative cascade) are active, the Fc portion of the antibodies initiates the cascade to form membrane lesions and ultimately lysis of the SRBs. The reaction was stopped by a 1-ml addition of EGTA-DGVB buffer. After pelleting the unlysed SRB cells, the degree of lysis was determined by the amount of hemoglobin released (absorbance at 414 nm). The total hemoglobin content was assessed by lysing cells in distilled water.

Lipoprotein Fractionation—The total lipoprotein fraction was separated from plasma by adjusting the plasma density to 1.25 g ml^{-1} before centrifuging at 114,000 × g_{av} using a Beckman Ti-60 rotor at 15 °C for 48 h.

Lipoprotein subfractions were isolated by sequential density centrifugations as reported in Wills *et al.* (1984). Briefly, a 1.006 g ml^{-1} solution (195 mM NaCl, 1 mM EDTA, 0.03% NaN_3) was carefully laid over plasma and spun at 114,000 × g_{av} using a Beckman Ti-60 rotor at 15 °C for 18 h. The upper layer containing a mixture of chylomicr and very low density lipoproteins (VLDL) was isolated using a tu slicer. The clear zone beneath this layer was removed down to the plasma volume and the plasma density was adjusted to 1.063 g ml^{-1} by the addition of NaBr. After a second centrifugation at 114,000 × g_{av} for 20 h, the low density lipoproteins (LDL) were isolated from the upper layer. The plasma density was then adjusted to 1.21 g ml^{-1} with NaBr and the solution spun for 48 h at 114,000 × g_{av}. High density lipoproteins (HDL) were isolated from the upper layer. The fractions were dialyzed against 200 volumes of 150 mM NaCl, 20 mM HEPES pH 7.4 and diluted to approximately their normal plasma concentrations based on the initial plasma volume and volume of each fraction.

Osmolarity Measurements—Solution osmolarities were determined by freezing point depression using an Osmette A Osmometer (Precision Systems Inc., Natick, MA). Standards (100, 240, and 900 mosm/kg) were analyzed prior to samples, which were measured at least in duplicate.

Analytical Procedures—In some experiments phospholipid concentrations were determined by phosphate assay (Fiske and Subbarow, 1925). Radiolabels were quantified by liquid scintillation counting using a Beckman LS3801 instrument.

RESULTS

We have shown previously that LUVs prepared using the extrusion technique and maintained under isoosmotic conditions are not spherical (Mui *et al.*, 1993). This morphology is likely a result of passage through the filter pores during preparation. In consequence, when such vesicles are exposed to an osmotic gradient at least part of the resulting influx of water can be accommodated by the vesicles rounding up, which maximizes their volume to surface area ratio. Only when the vesicles are spherical will further influx of water exert an osmotic pressure on the bilayer. The increase in vesicle volume resulting from this morphological transformation will have the effect of diluting the intravesicular solute; in order to calculate the actual osmotic differential experienced by the vesicles, this dilution effect must be taken into account. We therefore determined the increase in trapped volume resulting from this shape change for the POPC:cholesterol LUVs used in the present study. As described under "Materials and Methods," vesicles were prepared in 10 mM NaCl, 1 mM glucose (1.5 μCi ml^{-1} [^{3}H]glucose) and incubated at 45 °C either under isoosmotic conditions or exposed to a small osmotic gradient by adjusting the external salt concentration to 2 mM. The external solution in both cases contained 1 mM glucose (1.5 μCi ml^{-1} [^{3}H]glucose). Glucose is slowly membrane permeable with a half-time for equilibration at 45 °C of 1 h. At various times, trapped volumes were determined following passage of the vesicles down a Sephadex G-25 column to remove unencapsulated [^{3}H]glucose. As shown in Fig. 1, control vesicles maintained in 10 mM NaCl show little change in trapped volume over the 45-h incubation period. In contrast, vesicles allowed to round up exhibit a 40% increase in internal volume over the same period. In subsequent experiments, this value was used to determined the actual osmotic differential experienced by vesicles exposed to a

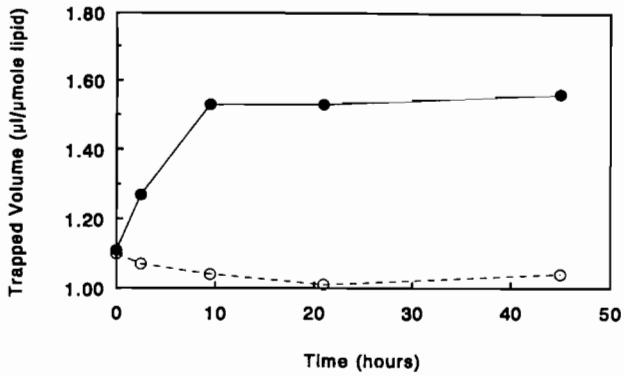

FIG. 1. **Influence of vesicle morphology on trapped volume.** Vesicles of POPC:cholesterol (50 mg ml^{-1}) were made in 10 mM NaCl, 1 mM glucose (1.5 µCi ml^{-1} [^{3}H]glucose) and diluted 5-fold with either 1 mM glucose (●) or 10 mM NaCl, 1 mM glucose (○) and incubated at 45 °C. Trapped volumes were measured at various times as described under "Materials and Methods."

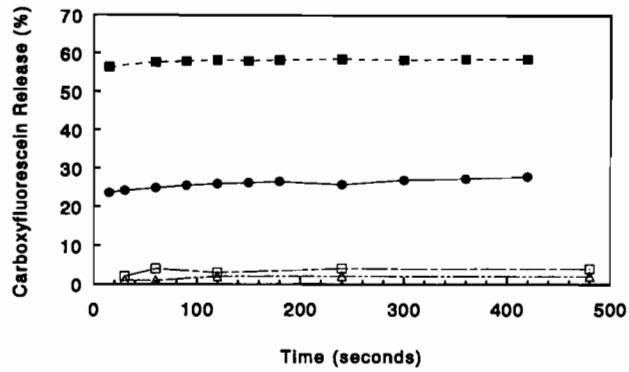

FIG. 2. **Time course of carboxyfluorescein release from osmotically stressed vesicles.** POPC:cholesterol vesicles (1700 mosm/kg internal osmolarity) were diluted 1:100 (final lipid concentration 60 µM) into hypoosmotic buffer (150 mM NaCl, 20 mM HEPES pH 7.4) in the presence (■) or absence of 10% plasma (●), or into isoosmotic buffer (1.4 M glucose, 150 mM NaCl, 20 mM HEPES pH 7.4) in the presence of 10% plasma (□). POPC:cholesterol vesicles made in isotonic 300 mosm/kg buffer (100 mM carboxyfluorescein, 20 mM HEPES pH 7.4) were also similarly diluted into 150 mM NaCl buffer in the presence of 10% plasma (△).

given applied osmotic gradient. As noted in our earlier study, the increase in trapped volume measured for vesicles exposed to a small osmotic gradient cannot be accounted for by swelling of initially spherical systems, which would generate a volume increase of less than 0.2% (Mui *et al.*, 1993).

The kinetics of carboxyfluorescein release from POPC:cholesterol LUVs in response to an osmotic shock are illustrated in Fig. 2. When vesicles prepared with an internal osmolarity of 1700 mosm/kg are diluted into 150 mM NaCl (300 mosm/kg), there is a rapid release (<10 s) of about 25% of the intravesicular carboxyfluorescein with little further loss up to 400 s. While the presence of 10% plasma greatly enhances the extent of carboxyfluorescein release, the kinetics are unchanged with essentially all of the loss occurring within the earliest measurable time point. This release profile is consistent with a transient rupture of the liposomal membrane. It should be noted that when vesicles are diluted into isoosmotic buffer (150 mM NaCl, 1.4 M glucose) containing 10% plasma, no significant carboxyfluorescein release could be observed (Fig. 2). In order to eliminate the possibility that high concentrations of glucose present in the isoosmotic buffer inhibited the plasma effect, vesicles prepared with an interior osmolarity of 300 mosm/kg were diluted into 150 mM NaCl, 10% plasma; again, very little carboxyfluorescein release is observed over the time period followed. We next examined how the magnitude of the applied osmotic gradient influenced solute release in the presence and absence of plasma. POPC:cholesterol LUVs prepared with an intravesicular solute osmolarity of 1700 mosm/kg were diluted into solutions of various osmolarities and the extent of carboxyfluorescein release monitored. As shown in Fig. 3, in the absence of plasma little fluorophore release is observed until an osmotic differential in excess of about 1100 mosm/kg is applied. As the differential is increased above this value, proportionately more carboxyfluorescein is lost from the vesicles. In the presence of 10% plasma, however, the threshold value for solute release is considerably reduced, to approximately 700 mosm/kg. Based on an initial internal osmolarity of 1700 mosm/kg and allowing for a 40% increase in trapped volume, we can calculate that an osmotic gradient of about 500 mosm/kg can be accommodated by the vesicles rounding up. Thus the actual minimum osmotic gradients the vesicles can withstand without lysis are 200 and 600 mosm/kg in the presence and absence of plasma, respectively.

The ability of plasma to lower the minimum osmotic gradient needed to initiate lysis suggests that a plasma component is able to interact with, and destabilize, the liposome membrane prior to the formation of major bilayer defects such as those

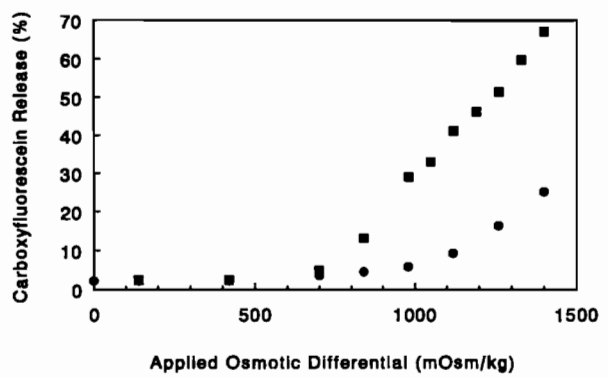

FIG. 3. **Influence of osmotic differential on vesicle lysis.** POPC:cholesterol vesicles (1700 mosm/kg internal osmolarity) were diluted 1:100 (final lipid concentration 60 µM) into solutions of varying osmolarity containing 150 mM NaCl, 20 mM HEPES pH 7.4 and 0 to 1.4 M glucose, either in the presence (■) or absence (●) of 10% plasma. After 2 min, the extent of carboxyfluorescein release was determined fluorometrically following dilution of an aliquot of this mixture 1:60 with a saline/glucose solution of the same osmolarity.

transiently created by lytic rupture. This interpretation is supported by the observation that if vesicles are diluted into a hypoosmotic buffer and plasma subsequently added, the extent of carboxyfluorescein release is the same as for LUVs osmotically shocked in the presence of plasma (data not shown).

After vesicle lysis the theoretical residual osmotic gradient can be calculated taking into account both the amount of solute released and the increase in trapped volume associated with rounding up. In Fig. 4 we show the calculated residual differential as a function of the applied osmotic gradient using carboxyfluorescein release data taken from Fig. 3. For applied osmotic gradients of less than about 500 mosm/kg, influx of water can be accommodated by the vesicles adopting a more spherical shape; the residual differential therefore will be zero. For applied osmotic gradients greater than 500 mosm/kg, however, influx of water into fully spherical LUVs will create an osmotic pressure. The *dashed line* in Fig. 5 represents the expected residual osmotic differential in the absence of any vesicle lysis. For vesicles exposed to osmotic gradients of varying magnitude in the absence of plasma, the residual differential approaches a limiting value of about 600 mosm/kg (Fig. 5). In the presence of 10% plasma, however, the vesicle residual

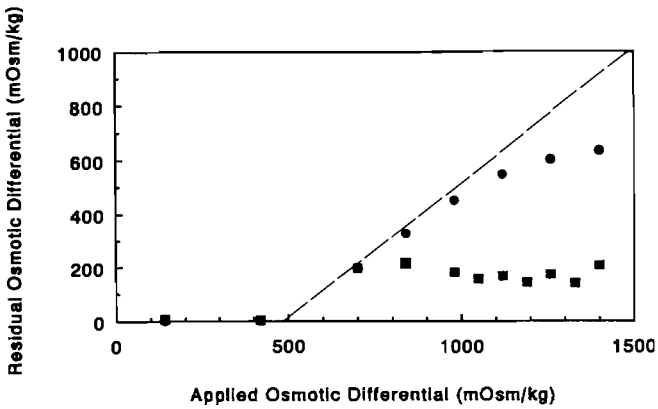

FIG. 4. **Calculated residual osmotic differential after vesicle lysis.** The amount of carboxyfluorescein released from POPC: cholesterol vesicles exposed to various hypoosmotic buffers in the presence (■) and absence (●) of 10% plasma (Fig. 3) was used to calculate the vesicles' residual osmotic differentials, taking into account an initial 40% increase in trapped volume due to the vesicles rounding up.

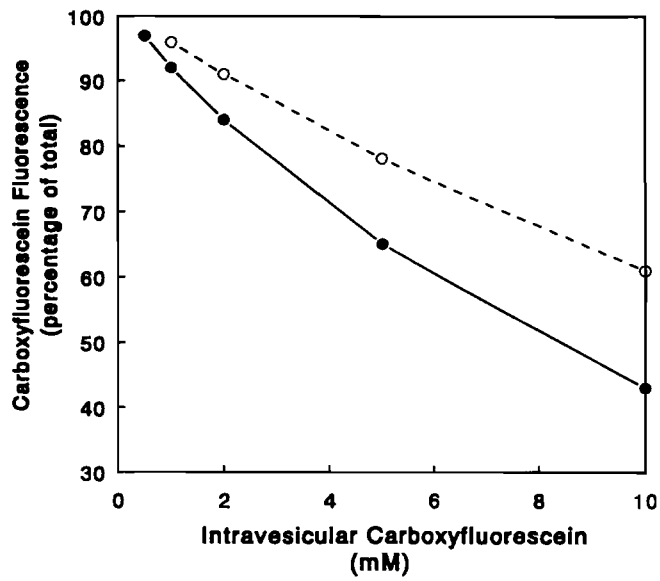

FIG. 5. **Quench curve of carboxyfluorescein entrapped in POPC: cholesterol vesicles.** Carboxyfluorescein fluorescence was determined in POPC:cholesterol vesicles containing 750 mM NaCl, 20 mM HEPES pH 7.4 (●) or 300 mM NaCl, 20 mM HEPES pH 7.4 (○) and 0.2–15 mM carboxyfluorescein. Total carboxyfluorescein fluorescence was determined by addition of octyl glucopyranoside (25 mM final concentration).

differential plateaus at about 200 mosm/kg. It is notable that, while the residual differentials in the presence and absence of plasma are very different, they are in each case in good agreement with corresponding values for the minimum osmotic gradient required to initiate lysis. In the case of vesicles osmotically shocked in the absence of plasma, we have shown previously that this correlation between the lysis threshold and residual differentials arises because vesicle lysis results in only partial release of intravesicular solute (Mui *et al.*, 1993). Earlier studies have reported that SUVs composed of dipalmitoylphosphatidylcholine when exposed to either HDL or apolipoprotein A-I lyse releasing all encapsulated solute (Weinstein *et al.*, 1981; Klausner *et al.*, 1985). We were therefore interested in determining whether osmotic lysis of LUVs in the presence of plasma results in only partial carboxyfluorescein release or whether it constitutes an "all-or-nothing" response similar to that reported by Weinstein and colleagues (1981). The experiment described below was performed to resolve this issue.

When carboxyfluorescein is encapsulated within LUVs it exhibits a steep concentration-dependent self-quenching between 0.5 and 10 mM (Fig. 5). This behavior is also influenced by the ionic strength of the medium with the degree of self-quenching being less pronounced at lower salt concentrations. We can take advantage of fluorophore quenching to distinguish between partial and total solute release following lysis in the presence of plasma, using a procedure similar to that described by Weinstein *et al.* (1981). The experimental rationale is that if lysis results in complete loss of intravesicular solute, then any carboxyfluorescein remaining entrapped must be in unlysed vesicles at its original concentration and, hence, initial level of quenching. Conversely, if all vesicles lyse releasing a portion of their contents only, then the level of fluorophore quenching will be reduced in direct proportion to the percentage of solute lost. Vesicles composed of POPC:cholesterol containing 10 mM carboxyfluorescein, 835 mM NaCl, 20 mM HEPES pH 7.4, and [^{14}C]citrate (5 μCi ml^{-1}) were hypoosmotically lysed in the presence of 10% plasma. Carboxyfluorescein and [^{14}C]citrate released were then removed by passage of the vesicles down a Bio-Gel A-1.5m column. As shown in Table I, two osmotic gradients of differing magnitude were examined, both of which were in excess of that needed to trigger lysis. At applied osmotic gradients of 700 and 1400 mosm/kg, [^{14}C]citrate and carboxyfluorescein are released to similar extents (Table I). Based on the fluorescence quenching determined following lysis, we can calculate the intravesicular carboxyfluorescein concentration using the quench curves shown in Fig. 5. This measured concentration can then be compared to concentrations predicted by the "partial release" or "all-or-nothing" models after correcting for trapped volume changes associated with rounding up. It is clear from Table I that the experimental data are consistent with the partial release model and imply that osmotic lysis of LUVs even in the presence of plasma does not result in complete solute loss. It should be noted that in this experiment vesicle swelling resulting from the applied osmotic gradients will not significantly contribute to the observed changes in fluorophore quenching. Based on a fractional increase in membrane area before failure (α_c) of 0.03 for stearoyloleoylphosphatidylcholine:cholesterol vesicles (Needham and Nunn, 1990), we can calculate a volume increase due to swelling of less than 5%, giving rise to a reduction in carboxyfluorescein quenching of less than 2%.

The size of the membrane defects created during osmotic lysis were probed by measuring the release of aqueous markers of differing size. In the presence and absence of plasma, both [^{14}C]citrate (M_r 192) and [^{3}H]dextran (average M_r 70,000, average hydrodynamic diameter 12 nm) are released to a similar extent (Table II). This is in agreement with the results of the previous experiment and would suggest that plasma components do not significantly alter the size of the lysis defect or interfere with resealing of the vesicle membrane following rupture.

We next examined the effect of plasma concentration on vesicle osmotic lysis. Fig. 6 shows the residual osmotic differential as a function of the applied osmotic gradient for POPC: cholesterol LUVs exposed to hypoosmotic solutions containing varying concentrations of plasma (0–10%). The *dashed line* represents the expected residual differential in the absence of lysis. There is clearly a plasma concentration-dependent reduction in the residual differential, and this effect is titratable with little additional increase in osmotic sensitivity above 5% plasma at the vesicle lipid concentration employed (60 μM).

Having characterized the influence of plasma on the osmotic sensitivity of LUVs, we next sought to identify the component or components responsible for this effect. In the following series of experiments, the influence of individual plasma constituents

TABLE I
Evaluation of theoretical models of solute release during plasma-enhanced lysis

Applied osmotic differential	Retained		Fluorescence	Intravesicular [CF]		
				Theoretical		Measured[c]
	[^{14}C]Citrate	CF		Total[a]	Partial[b]	
mosm/kg	%		%		*mM*	
0	100	100	43	10	10	10
700	87	92	58	7.1	6.4	6.5
1400	53	56	81	7.1	3.9	4.1

[a] Theoretical intravesicular carboxyfluorescein concentration based on a model in which total solute release occurs from vesicles during lysis and hence any retained carboxyfluorescein is in unlysed vesicles. The calculation is based on an initial carboxyfluorescein concentration of 10 mM and assumes a 40% maximum increase in vesicular volume due to rounding up.

[b] Theoretical intravesicular carboxyfluorescein concentration based on a model in which lysis results in partial solute release from all vesicles in the population. Again the calculation is based on an initial carboxyfluorescein concentration of 10 mM and assumes a maximum 40% increase in vesicular volume due to rounding up.

[c] Determined from Fig. 5 based on the measured fluorescence quenching.

TABLE II
Influence of solute molecular weight on release during vesicle lysis in the presence of plasma

	% Release[a]	
	[^{14}C]Citrate	[^{3}H]Dextran
Buffer	24 ± 8	20 ± 10
10% plasma	39 ± 8	30 ± 10

[a] POPC:cholesterol vesicles were subjected to hypoosmotic lysis in the presence and absence of 10% plasma as described under "Materials and Methods." The measurements are from an average of six trials, and the errors represent one standard deviation.

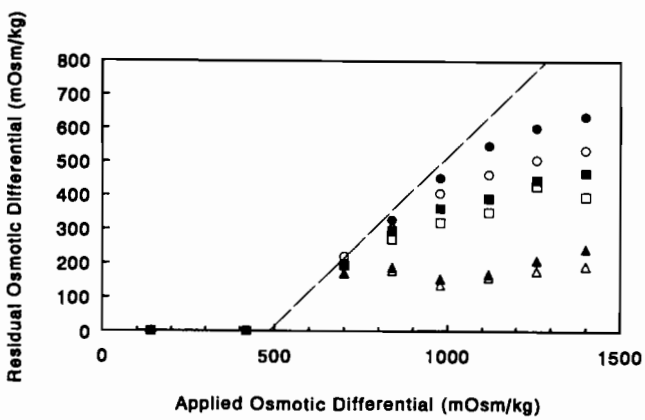

FIG. 6. **Influence of plasma concentration on vesicle lysis.** POPC:cholesterol vesicles (1700 mosm/kg internal osmolarity, final lipid concentration 60 μM) were exposed to various hypoosmotic buffers containing 0% (●), 0.02% (○), 0.1% (■), 0.2% (□), 5% (▲), and 10% plasma (△). After a 2-min incubation at 23 °C, the amount of carboxyfluorescein released was measured and the residual osmotic differential calculated.

on vesicle lysis was examined. As the most abundant protein present in plasma, albumin was studied first. Fig. 7*A* shows that the presence of 0.5% fatty acid-free bovine serum albumin has only a modest influence on vesicle lysis. Given that this bovine serum albumin concentration is equivalent to that present in 10% plasma, it is clear that at lower plasma concentration, where the potentiation of vesicle lysis is still almost maximal, the contribution to this effect by albumin will be negligible. A second plasma constituent that might be anticipated to destabilize the liposome membrane would be the complement system. This system plays an important role in defense against foreign organisms. Once activated, complement proteins can insert into the target membrane creating pores and hence triggering lysis of the organism. To determine whether this system is involved in potentiating vesicle lysis, complement proteins were inactivated by heating serum at 56 °C for 30 min. In this experiment, serum was used instead of plasma due to the need to include Mg^{2+} in the hemolytic assay

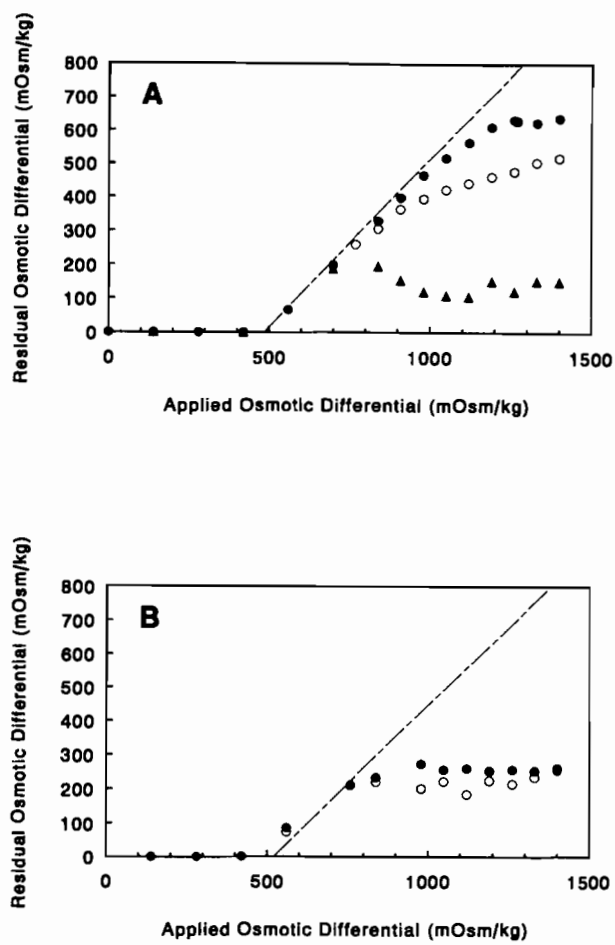

FIG. 7. **Influence of albumin and complement-inactivated serum on vesicle lysis.** *A*, POPC:cholesterol vesicles (1700 mosm/kg internal osmolarity, final lipid concentration 60 μM) were exposed to various hypoosmotic buffers (●) or hypoosmotic buffers containing 10% plasma (▲) or 0.5% bovine serum albumin (○). *B*, POPC:cholesterol vesicles (1700 mosm/kg internal osmolarity, final lipid concentration 60 μM) were exposed to hypoosmotic buffers in the presence of complement-inactivated (○) or normal (●) serum. As described under "Materials and Methods," the plasma proteins necessary for complement activity were denatured by incubating serum at 56 °C for 30 min.

employed to determine complement activity. While incubation at 56 °C eliminated complement activity, as shown in Fig. 7*B*, little difference was observed between heat-inactivated and normal serum with respect to its ability to potentiate vesicle osmotic lysis.

Several lipid species, including cholesterol, fatty acids, and lysophospholipids, are readily able to exchange between mem-

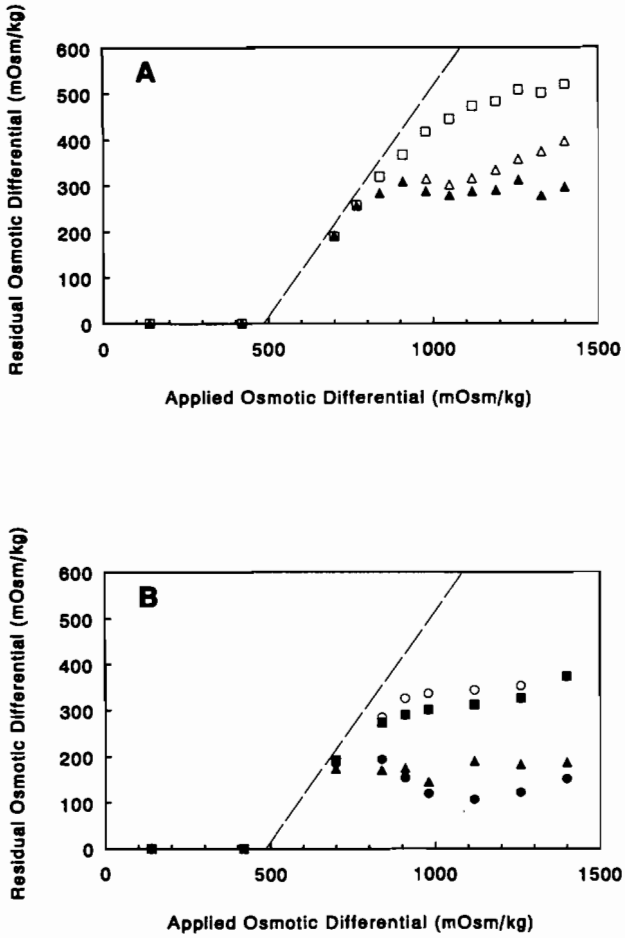

FIG. 8. **Influence of lipoproteins on vesicle lysis.** *A*, lipoproteins were fractionated from plasma by density centrifugation as described under "Materials and Methods." The infranatant and lipoprotein-containing supernatant were then diluted to their normal plasma concentrations, based on the total plasma volume and the volume of the two fractions. The ability of 0.2% infranatant (□) and 0.2% lipoprotein (△) fractions to potentiate lysis of POPC:cholesterol vesicles (60 μM phospholipid) were compared to 0.2% plasma (▲). *B*, influence of VLDL plus chylomicron, LDL, and HDL fractions on vesicle lysis. The various lipoproteins were fractionated by sequential density centrifugation and diluted back to their normal plasma concentrations as described under "Materials and Methods." The ability of these fractions to potentiate osmotic lysis was then determined by exposing POPC:cholesterol vesicles (60 μM phospholipid) to hypoosmotic buffers containing the equivalent of 10% VLDL plus chylomicron (○), LDL (■), and HDL (▲) fractions, as well as 10% whole plasma (●).

branes. Consequently incubation of POPC:cholesterol LUVs with plasma might alter the lipid composition of the vesicles, resulting in the observed enhanced susceptibility to osmotic lysis. The LUVs used in this study were prepared with a cholesterol content (45 mol %) that should minimize any net stearol transfer upon incubation in plasma (Cooper *et al.*, 1975). Migration of fatty acid or lysophospholipid from chylomicrons, albumin, or lipoproteins to the vesicles would, however, be expected; therefore, we examined the influence of these lipids on the osmotic properties of POPC:cholesterol LUVs. The incorporation of oleic acid (0–8 mol %) or monooleoylphosphatidylcholine (0–10 mol %) into POPC:cholesterol vesicles, however, did not increase their susceptibility to osmotic lysis (results not shown).

We next turned our attention to the lipoproteins and, using density gradient centrifugation, separated a total lipoprotein fraction from whole plasma. When diluted to a concentration equivalent to that in plasma, this lipoprotein fraction enhanced vesicle osmotic lysis to approximately the same extent as whole plasma (Fig. 8*A*). In contrast the lipoprotein-depleted plasma exhibited similar properties to those of albumin alone (*cf.* Fig. 7*A*). Further fractionation of the total lipoprotein pool using density gradient centrifugation yielded the chylomicrons plus VLDL, LDL, and HDL. Each fraction was diluted back to its normal plasma concentration, and its influence on osmotic lysis was then assayed. While all three fractions enhance lysis, as shown in Fig. 8*B*, HDL had the most pronounced effect and was in fact comparable to whole plasma.

DISCUSSION

The results presented here have important implications both with respect to the interaction of plasma components with lipid vesicles and relating to the application of liposomes as systemic delivery vehicles for therapeutic agents. These two areas will be discussed in turn.

It is clear that plasma dramatically increases the osmotic sensitivity of POPC:cholesterol vesicles with the membrane tension at lysis being reduced from about 36 to about 12 dynes/cm. Despite this increased sensitivity, however, the characteristics of lysis in either the presence or absence of plasma remain similar. In both cases, for example, solute loss is relatively independent of molecular weight, at least for compounds of less than about 70 kilodaltons. Similarly, lysis is not an all-or-nothing process but instead results in only partial loss of intravesicular solute with the percentage released depending upon the magnitude of the osmotic gradient. In both the presence and absence of plasma, therefore, following bilayer resealing, the intravesicular solution remains hypertonic with respect to the external medium.

As discussed in an earlier paper (Mui *et al.*, 1993), the observation that the minimum osmotic differential required to trigger lysis is of similar magnitude to the residual gradient following lysis can be more easily accounted for by a model that assumes solute release occurs in a multi-step process. It is very unlikely that a single lytic event would, fortuitously, release sufficient intravesicular solute such that the remaining osmotic differential was just below the threshold needed to produce further lysis. It is our belief, therefore, that on exposure of large unilamellar vesicles to a relatively large osmotic differential, water influx generates an osmotic pressure resulting in bilayer rupture. While this allows temporary dissipation of the hydrostatic pressure, only a small fraction of the intravesicular solute is lost before the bilayer reseals. The intravesicular solution will remain hyperosmotic, therefore, resulting in further influx of water, subsequent membrane rupture, and additional solute loss, followed again by membrane resealing. This cycle will continue until sufficient intravesicular solute has been released such that the lipid bilayer is able to withstand the osmotic pressure resulting from the residual osmotic differential. Plasma, we contend, decreases the strain tolerance of the vesicle membrane necessitating additional cycles of swelling and rupture before the bilayer can tolerate the residual differential.

While our observation that osmotic lysis in the presence of plasma results in only partial solute release appears to be at variance with earlier work (Weinstein *et al.*, 1981), the two experimental systems are very different. Weinstein and coworkers observed that interaction of serum lipoproteins or apolipoproteins with SUVs composed of dipalmitoylphosphatidylcholine could induce complete release of entrapped carboxyfluorescein in an all-or-nothing manner. In contrast to the present study, however, solute release was triggered by heating the vesicles through their gel to liquid-crystalline transition temperature. In this system protein interaction, and presumably solute release, is believed to occur at the lipid phase

domain boundaries. Furthermore, the authors noted that the characteristics of phase transition release were dependent on the type of liposome studied, large multilamellar, and large unilamellar vesicles exhibiting different behavior from that displayed by small unilamellar systems (Weinstein *et al.*, 1981).

The ability of plasma to increase vesicle osmotic sensitivity does not arise from a nonspecific protein interaction. Albumin and other soluble plasma proteins, including those associated with the complement system, exert only a modest influence on the osmotic behavior of large unilamellar vesicles. In contrast, all of the lipoprotein fractions examined lowered vesicle tolerance to osmotic pressure, with high density lipoproteins exhibiting the most pronounced effect. This observation is consistent with earlier studies, which examined plasma- or serum-induced solute leakage from liposomes and which identified lipoproteins as playing a major role in this process (Comiskey and Heath, 1990). A number of studies have shown that lipoproteins can mediate exchange and/or transfer of phospholipids from liposomes (Scherphof *et al.*, 1984). We do not consider it likely, however, that this process significantly contributes to the increased osmotic sensitivity observed in the presence of plasma. As shown in Fig. 2, when cholesterol-containing vesicles are diluted into a hypoosmotic solution containing plasma, lysis with partial release of intravesicular solute is seen within the earliest measurable time point (15 s) with little further release over the time course followed. Based on earlier studies of phospholipid transfer between cholesterol-containing liposomes and high density lipoproteins, however, we would not expect significant lipid migration over the time frame during which lysis occurs (Damen *et al.*, 1981). As mentioned in the Introduction, lipoprotein-vesicle association appears to be influenced by the lipid packing density. This would be anticipated if protein interaction involved insertion of a hydrophobic or amphipathic domain into the bilayer. In turn this provides a rationale for the increased osmotic sensitivity exhibited by vesicles in the presence of plasma. Upon exposure of vesicles to a hypoosmotic medium, there will be a net influx of water creating a hydrostatic pressure. This outward pressure will cause elastic stretching of the membrane, and the resulting increase in area per lipid molecule will favor protein insertion. If this penetration disrupts bilayer cohesive interactions, vesicle rupture will occur. While this interpretation must be considered speculative, support for the general principle is provided by a recent study that examined the ability of certain amphiphiles to selectively interact with, and disrupt, vesicles subjected to osmotic stress (Naka *et al.*, 1992). This study compared the ability of several novel surfactants to elicit release of carboxyfluorescein from large unilamellar vesicles maintained under isoosmotic conditions or subjected to a hypotonic medium. Two of the compounds tested were lytic only when vesicles were osmotically stressed suggesting that bilayer penetration, and subsequent disruption, occurred only when the membrane was stretched and the lipid packing density therefore reduced. One prediction arising from our proposed mechanism of action is that plasma-induced osmotic sensitivity should be dependent upon vesicle lipid composition. Clearly lipid mixtures that minimize protein interaction with, and or insertion into, the bilayer should be less sensitive to plasma-induced lysis.

The present study also has practical implications with respect to the use of liposomes as drug delivery vehicles. A number of studies have demonstrated that, following intravenous administration, liposomes preferentially accumulate at sites of inflammation and disease, including tumor sites, and tend not to be deposited in healthy organs such as the heart and kidney (for a review, see Ostro and Cullis (1989)). As a consequence, liposomal encapsulation can reduce the toxic side effects of certain drugs on normal tissue, while maintaining or enhancing drug efficacy. In many cases maximizing the benefits of liposomal delivery requires that the drug remains encapsulated until its carrier either accumulates at the disease site or is cleared from the circulation. For a wide variety of pharmaceutical agents, efficient liposomal uptake at high drug-to-lipid ratios can be achieved employing vesicles exhibiting a transmembrane pH gradient, interior acidic (Madden *et al.*, 1990). Drugs that are weak bases will redistribute between the intravesicular solution and the external medium in accordance with the Hendersen-Hasselbach equation. This uptake process, however, consumes intravesicular protons as accumulated drug is protonated at the vesicle interior. In order to maintain the pH gradient and maximize drug uptake therefore, it is important that the intravesicular medium provide a large buffering capacity. For such systems the final drug concentration encapsulated is (theoretically) dependent only on the buffer concentration of the intravesicular solution. We show here, however, that the osmotic sensitivity of liposomes, particularly in the presence of plasma, places constraints on the osmolarity of the intravesicular medium. Clearly, given the objective identified above that a liposomal drug should remain associated with its carrier, the intravesicular buffering capacity, and hence osmolarity, should be selected so as to ensure that osmotic lysis does not occur upon exposure to plasma.

REFERENCES

Allen, T. M., and Cleland, L. G. (1980) *Biochim. Biophys. Acta* **597,** 418–426

Allen, T. M., and Everest, J. M. (1983) *J. Pharmacol. Exp. Ther* **226,** 539–544

Allen, T. M., Ryan, J. L., and Papahadjopoulos, D. (1985) *Biochim. Biophys. Acta* **818,** 205–210

Allen, T. M., Mehra, T., Hansen, C., and Chin, Y. C. (1992) *Cancer Res.* **52,** 2431–2439

Comiskey, S. J., and Heath, T. D. (1990) *Biochemistry* **29,** 3626–3631

Cooper, R. A., Arner, E. C. Wiley, J. S., and Shattil, S. J. (1975) *J. Clin. Invest.* **55,** 115–126

Damen, J., Regts, J., and Scherphof, G. (1981) *Biochim. Biophys. Acta* **665,** 538–545

Epand, R. M., Surewicz, W. K., Hughes, D. W., Mantsch, H., Segrest, J. P., Allen, T. M., and Anantharamaiah, G. M. (1989) *J. Biol. Chem.* **264,** 4628–4635

Evans, E. A., and Needham, D. (1987) *J. Phys. Chem.* **91,** 4219–4228

Fiske, C. H., and Subbarow, Y. (1925) *J. Biol. Chem.* **66,** 375–400

Hope, M. J., Bally, M. B., Webb, G., and Cullis, P. R. (1985) *Biochim. Biophys. Acta* **812,** 55–65

Kanellis, P., Romans, A. Y., Johnson, B. J., Kercret, H., Chiovetti, R., Jr., Allen, T. M., and Segrest, J. P. (1980) *J. Biol. Chem.* **255,** 11464–11472

Kirby, C., Clarke, J., and Gregoriadis, G. (1980) *Biochem. J.* **186,** 591–598

Klausner, R. D., Blumenthal, R. Innerarity, T., and Weinstein, J. N. (1985) *J. Biol. Chem.* **260,** 13719–13727

Lelkes, P. I., and Tandeter, H. B. (1982) *Biochim. Biophys. Acta* **716,** 410–419

Madden, T. D., Harrigan, P. R., Tai, L. C. L., Bally, M. B., Mayer, L. D., Redelmeier, T. E., Loughrey, H. C., Tilcock, C. P. S., Reinish, L. W., and Cullis, P. R. (1990) *Chem. Phys. Lipids* **53,** 37–46

Mayer, L. D., Hope, M. J., Cullis, P. R., and Janoff, A. S. (1985) *Biochim. Biophys. Acta* **817,** 193–196

Mui, B., Cullis, P. R., Evans, E. A., and Madden, T. D. (1993) *Biophys. J.* **64,** 443–453

Naka, K., Sadownik, A., and Regen, S. L. (1992) *J. Am. Chem. Soc.* **114,** 4011–4013

Needham, D., and Nunn, R. S. (1990) *Biophys. J.* **58,** 997–1009

Ostro, M. J., and Cullis, P. R. (1989) *Am. J. Hosp. Pharm.* **46,** 1576–1587

Pownall, H. J., Massey, J. B., Kusserow, S. K., and Gotto, A. M. (1979) *Biochemistry* **18,** 574–579

Rahman, A., Kessler, A., More, N., Sikic, B., Rowden, G., Woolley, P., and Schein, P. S. (1980) *Cancer Res.* **40,** 1532–1537

Scherphof, G., and Morselt, H. (1984) *Biochem. J.* **221,** 423–429

Scherphof, G., Roerdink, F., Waite, M., and Parks, J. (1978) *Biochim. Biophys. Acta* **542,** 296–307

Scherphof, G. L., Damen, J., and Wilschut, J. (1984) in *CRC Liposome Technology* (Gregoriadis, G., ed) Vol. 3, pp. 205–224, CRC Press, Boca Raton, FL

Weinstein, J. N., Klausner, R. D., Innerarity, T., Ralston, E., and Blumenthal, R. (1981) *Biochim. Biophys. Acta* **647,** 270–284

Weinstein J. N., Ralston, E., Leserman, L. D., Klausner, R. D., Dragsten, P., Henkart, P., and Blumenthal, R. (1984) in *CRC Liposome Technology* (Gregoriadis, G., ed) Vol. 3, pp. 183–204, CRC Press, Boca Raton, FL

Whaley, K. (ed) (1985) in *Methods in Complement for Clinical Immunologists*, pp. 77–139, Churchill Livingstone, Edinburgh

Wills, G. L. Lane, P. A., and Weech, P. K. (1984) in *A Guidebook to Lipoprotein Technique: Laboratory Techniques in Biochemistry and Molecular Biology* (Burdon, R. H., and van Krippenberg, P. H., eds) Vol. 14, p. 18, Elsevier, Amsterdam