Small, but Not Large, Unilamellar Liposomes Composed of Dioleoylphosphatidylethanolamine and Oleic Acid Can Be Stabilized by Human Plasma†

Dexi Liu and Leaf Huang*
Department of Biochemistry, University of Tennessee, Knoxville, Tennessee 37996-0840
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ABSTRACT: Small unilamellar liposomes, composed of dioleoylphosphatidylethanolamine (DOPE) and oleic acid (OA), prepared by sonication, were incubated in the presence of human plasma at 37 °C. The release of entrapped calcein after 8-h incubation was about 15% in plasma, compared with about 70% in phosphate-buffered saline under the same conditions. In contrast, dioleoylphosphatidylcholine (DOPC)/OA liposomes under the same conditions release about 70% in plasma and only 10% in PBS. Total release of calcein from the DOPE/OA liposomes was observed in a PBS solution containing bovine serum albumin, and the release was completely blocked by preincubation of the liposomes with plasma. These results indicate that the unstable DOPE/OA liposomes are stabilized by incubation with plasma. The stabilization process was very fast, being completed within 1 min. Only relatively small liposomes ($d \leq 200$ nm) were completely stabilized by plasma; larger liposomes were progressively less stabilizable. SDS–polyacrylamide gel electrophoresis of liposomes which had been incubated with plasma and then washed indicated that several proteins were tightly associated with liposomes. Using liposomes containing [^{14}C]OA, it was found that about 70% of the original OA was extracted after 1-h incubation with human plasma at 37 °C. Thin-layer chromatographic analysis of the plasma-treated liposomes showed the presence of the plasma lipids in the liposomes. These results suggest that liposomes composed of DOPE/OA are stabilized by protein and/or lipid components from human plasma and that the composition of the liposomes is altered. The mechanism of stabilization is discussed in terms of the surface pressure of small vesicles with a high degree of curvature. The results presented here are relevant to the use of PE-containing liposomes for drug delivery in vivo.

Liposomes have been extensively tested in experimental animals and in humans as carriers for drugs, enzymes, biological response modifiers, and nucleic acids [for a recent review, see Gregoriadis (1988)]. Of primary concern is the stability of the liposome during storage and in the blood. The former often limits the clinical use of the liposome, and the latter determines the carrier potential of the liposome. It is well-known that liposomes composed of phosphatidylcholine (PC)[1] as the major matrix lipid are generally stable in a simple buffer upon storage (Szoka, 1980), and this type of liposome is widely used by many investigators. However, unsaturated PC-based liposomes rapidly release the entrapped contents upon exposure to serum or plasma, unless cholesterol is included as one of the major lipid components (Gregoriadis & Senior, 1980). Plasma components such as albumin, lipoproteins, and fibronectin readily interact with the liposomal bilayer (Juliano & Lin, 1980). Lipoproteins such as HDL are particularly important in determining liposome stability in the blood (Kirby et al., 1980). Apolipoproteins are transferred to liposome membranes (Luke et al., 1980; Williams & Scanu, 1986) and perhaps serve as opsonins for the uptake of liposomes by macrophages (Ivanov et al., 1985). Different sets of serum proteins are found to interact with liposomes depending on the cholesterol content of the liposomes (Moghimi & Patel, 1988).

Liposomes composed predominantly of PE, and particularly an unsaturated PE such as DOPE, have become increasingly important for the liposomal drug delivery in recent years (Ellens et al., 1984; Straubinger et al., 1985; Wang et al., 1986; Connor et al., 1984; Collins & Huang, 1987; Wang & Huang, 1987; Connor & Huang, 1986). The equilibrium phase of DOPE at physiological temperature and pH is the hexagonal H_{II} phase (Cullins & de Kruijff, 1979). However, the bilayer phase, in the form of liposomes, can be prepared by mixing DOPE with at least one other amphiphile (lipid or protein) (Ellens et al., 1984; Straubinger et al., 1985; Connor et al., 1984; Hu et al., 1986; Ho et al., 1986). These liposomes are generally less stable than PC-based liposomes upon storage due to their tendency to revert to the H_{II} phase. Indeed, special conditions such as acidic pH, or binding to target cells, often trigger a rapid destabilization of the liposomes, making the liposomes suitable for intracellular drug delivery (Collins & Huang, 1987; Wang & Huang, 1987; Connor & Huang, 1986; Ho et al., 1987a). Thus, DOPE liposomes stabilized with weakly acidic amphiphiles, such as fatty acids (Straubinger et al., 1985; Collins & Huang, 1987; Wang & Huang, 1987; Connor & Huang, 1986), acylamino acids (Connor et al., 1984; Connor & Huang, 1985), and other double-chain lipids (Leventis et al., 1987), are useful for efficient cytoplasmic delivery of drugs. DOPE liposomes stabilized with acylated antibody, known as target-sensitive immunoliposomes (Ho et al., 1986), are useful for cell surface drug delivery (Ho et al., 1987a) and in vitro diagnosis of virus (Ho et al., 1987b). At least one such liposome preparation, i.e., the large unilamellar liposomes composed of DOPE/OA (8:2), is not stable in serum; rapid liposome aggregation and content leakage take

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* Author to whom correspondence should be addressed.

[1] Abbreviations: chol, cholesterol; DOPE, dioleoylphosphatidylethanolamine; PC, phosphatidylcholine; DOPC, dioleoylphosphatidylcholine; OA, oleic acid; PBS, phosphate-buffered saline; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; PE, phosphatidylethanolamine; BSA, bovine serum albumin; TLC, thin-layer chromatography; DOC, deoxycholic acid.

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place within minutes after exposure to serum (Connor et al., 1986). Inclusion of cholesterol in such liposomes improves the stability (Liu & Huang, 1989). Thus, the behavior of large unilamellar liposomes composed primarily of DOPE is similar to that of liposomes composed primarily of PC.

We report here the observation that the small unilamellar liposomes of the same lipid composition, i.e., DOPE and OA, though quite unstable in buffer, become rapidly stabilized upon exposure to plasma or serum. We have isolated the stabilized liposomes and characterized their composition. Furthermore, we have shown that the plasma stabilization of liposomes depends on the liposome size.

Experimental Procedures

Materials. DOPE and DOPC were purchased from Avanti Polar Lipids. OA, cholesterol, BSA, ficoll, and calcein were obtained from Sigma Chemical Co. Freshly collected human plasma was obtained from Fort Sanders Regional Medical Center, Knoxville, TN. [^{14}C]Oleic acid (57 mCi/mmol) was purchased from New England Nuclear. TLC plates were from Beckman.

Preparation of Small Unilamellar Liposomes. Ten micromoles of lipids containing either DOPE or DOPC (6.67 μmol) and OA (3.33 μmol) was dried in a stream of N_2 gas. The dry lipids were vacuum-desiccated for a minimum of 30 min and hydrated for 5 h at room temperature in 1 mL of 50 mM calcein. The osmolarity of this solution was adjusted to 320 mOsm/kg with 10× of PBS, pH 8.0. A trace amount of a nonexchangeable lipid marker, hexadecyl [^{3}H]cholestanyl ether, was included to monitor the lipids. The lipid suspension was sonicated with a bath sonicator (Laboratory Supplies, Hicksville, NY), and the pH was adjusted and maintained at 8.0 during sonication. The liposome suspension was left for 2 h at room temperature to facilitate the annealing process. Free calcein was then separated from liposomes by using Bio-Gel A-0.5M column chromatography using PBS which was isotonic to the calcein-containing buffer as the eluent. The size of liposomes was measured by dynamic laser light scattering using a Coulter N4SD instrument. Liposomes with a diameter of 95 ± 10 nm were used for most of the experiments.

Liposome Stability Assay. Fifty microliters of the liposome suspension containing about 50–150 nmol of lipids was added to 450 μL of prewarmed (37 °C) human plasma or PBS (pH 7.4). Ten microliters of the mixture was added into a cuvette containing 1.99 mL of PBS for fluorescence measurement with constant stirring. The fluorescence intensity was measured with an excitation wavelength of 490 nm and an emission wavelength of 520 nm. The total fluorescence intensity was obtained by adding 50 μL of 5% deoxycholate to lyse the liposomes. Release of the entrapped calcein from the liposomes was calculated with the equation:

$$\% \text{ release} = \frac{(F_t/F)(F_x - F_0)}{F_t - F_0} \quad (1)$$

where F_0 is the fluorescence intensity of the liposomes in PBS at room temperature at time zero. F_t is the total fluorescence intensity of liposome in PBS after addition of DOC. F_x is the fluorescence intensity of the liposome incubated either in plasma or in PBS at 37 °C at time x. F is the total fluorescence intensity of the liposomes in plasma after addition of DOC. F_t/F is a correction term which is used to minimize the pipetting and other systematic errors in the measurement. The value of F_t/F ranged from 0.9 to 1.1.

Treatment of Liposomes with Plasma. Liposome suspensions containing 1 μmol of lipids (in 100 μL) were incubated with plasma of various dilutions for 1 h at 37 °C. Excess plasma proteins were separated from liposomes either by floating the liposomes in a discontinuous Ficoll gradient as described below or by chromatography on a Bio-Gel A-0.5M column.

pH Sensitivity Assay. Ten microliters of liposomes containing about 3 nmol of lipid was incubated in a cuvette containing 1.99 mL of PBS at the desired pH for 5 min before the pH was returned to 8.0 by adding appropriate amounts of NaOH. Equation 1 was used to calculate percent calcein release, except that F_0 was the fluorescence intensity at pH 8.0 and F_x was the fluorescence intensity at pH 8.0 after incubation at different pHs. F_t and F were the total fluorescence intensity after lysis of liposomes incubated at pH 8.0 and desired pHs with DOC.

Preparation of Liposomes of Various Diameters. Large unilamellar vesicles were prepared by a detergent dialysis method. Ten micromoles of lipid was hydrated in 900 μL of buffer containing 50 mM calcein and sonicated as described above. One hundred microliters of octyl glucoside (100 μmol) was added after sonication and pH adjustment. The mixture was vortexed and dialyzed at room temperature against 100 mL of the same buffer containing 1 g of washed SM-2 beads for 48 h with slow stirring. The liposomes were extruded through polycarbonate filters of defined pore size (manufactured by Nucleopore Corp.) to obtain unilamellar liposomes of defined size. Free calcein was then removed by gel filtration on a Bio-Gel A-0.5 M column as above. The size of the liposomes was measured by dynamic laser light scattering as above.

BSA-Induced Leakage of DOPE/OA Liposomes. The same amount of liposome as in the stability assay was incubated with 450 μL of BSA with 22, 44, or 88 mg/mL for 15 min at 37 °C. Ten microliters of the mixture was added to a cuvette containing 1.99 mL of PBS. The release was calculated according to eq 1.

Size Change of DOPE/OA Liposomes after Sonication. Liposomes (20 mM) were mixed either with an equal volume of PBS or with human plasma after sonication, and the size was measured either by dynamic laser light scattering as described above or by negative-stain electron microscopy using 1% uranium acetate as a stain as described previously (Wang & Huang, 1984).

Association of Plasma Components with Liposomes. Liposomes containing 5 μmol of lipids (in 0.5 mL) were incubated with 0.5 mL of human plasma at 37 °C for 1 h. The mixture was then mixed with a Ficoll solution (in PBS, pH 8.0) to obtain a final Ficoll concentration of 20% and placed into a 15-mL Corex centrifuge tube. Six milliliters of 15% Ficoll was overlayed, followed by 0.5 mL of PBS. The mixture was centrifuged in a Sorvall HB4 swinging-bucket rotor for 1 h at 5000 rpm at room temperature. The liposomes which floated to the PBS/15% Ficoll interface were collected. The resuspended liposomes were floated 1 more time with an identical procedure before being used for protein determination by the Lowry assay (Lowry et al., 1951) and for the SDS–PAGE analysis using a 7.5% polyacrylamide under denaturing conditions (Laemmli, 1970). The lipid was extracted from floated liposomes by using standard methods (Kates, 1986), and the amount of lipid was determined by a chromate assay (Suito & Sato, 1966) using DOPE/OA (2:1) as standard. The recovery of lipid was determined by using [^{3}H]cholestanyl ether as a trace marker. The lipid extract was also analyzed by thin-layer chromatography on a silica gel plate using chloroform/acetone/methanol/acetic acid/H_2O (6:8:2:2:1) as a developing solvent.

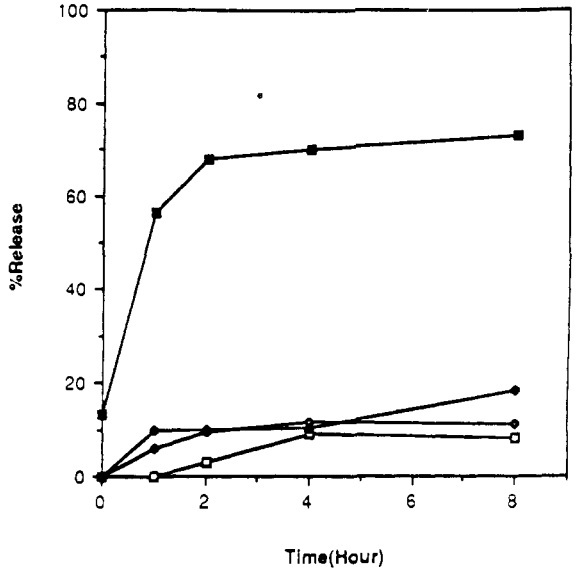

FIGURE 1: Stability of PC-based liposomes (150 nmol of total lipid) in 90% plasma or PBS at 37 °C. (□) DOPC/OA (2:1) in PBS; (◇) DOPC/OA/chol (2:1:2) in PBS; (■) DOPC/OA (2:1) in plasma; (◆) DOPC/OA/chol (2:1:2) in plasma.

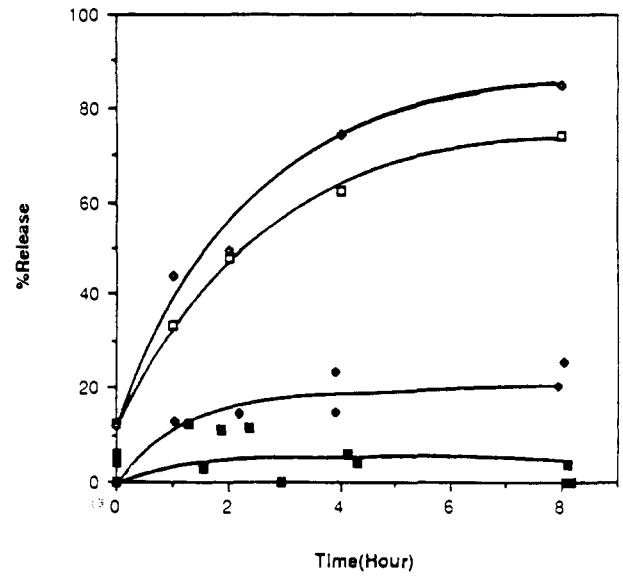

FIGURE 2: Stability of PE-based liposomes (150 nmol of total lipid) in 90% plasma or PBS at 37 °C. (□) DOPE/OA (2:1) in PBS; (◇) DOPE/OA/chol (2:1:2) in PBS; (■) DOPE/OA (2:1) in plasma; (◆) DOPE/OA/chol (2:1:2) in plasma.

The dissociation of oleic acid from liposomes was determined by using a trace amount of [^{14}C]OA with the same floatation procedure as described above. After one floatation, the gradient was fractionated from the bottom, and ^{14}C cpm of the fractions was determined. The ^{14}C cpm at bottom fractions was considered as the dissociated OA. The dissociation of OA was confirmed by gel filtration on a Bio-Gel A-1.5M column (1.4 × 60 cm) and by sucrose gradient centrifugation. The [^{3}H]cholestanyl ether was used as a lipid marker in these experiments.

RESULTS

Stability of the Small Sonicated Liposomes in Buffer and Plasma. Stability of liposomes was measured by the release of a water-soluble, self-quenching fluorescence dye, calcein, from the liposomes. When liposomes composed of DOPC/OA (2:1) or DOPC/OA/chol (2:1:2) were incubated in PBS at 37 °C, little calcein was released up to 8 h (Figure 1). However, when the incubation was carried out in 90% human plasma, cholesterol-free liposomes rapidly released calcein (approximately 70% after 2 h). The cholesterol-rich liposomes, on the other hand, were quite stable in the plasma, showing little calcein release even after 8 h. Therefore, the PC-based liposomes are stable in buffer and unstable in plasma unless cholesterol is present in the liposome membrane.

The stability of the corresponding PE-based liposomes was also studied (Figure 2). Small unilamellar liposomes composed of DOPE/OA (2:1) or DOPE/OA/chol (2:1:2) were not stable in PBS; continuous calcein release was observed up to 70–80% in 8 h. Inclusion of cholesterol in the liposome did not stabilize the liposome. Interestingly, calcein release from either type of liposome was not observed when the liposomes were incubated in 90% plasma. Inclusion of cholesterol made little difference in terms of the stability in plasma. Thus, the behavior of the PE-based liposome is just opposite to that of the PC-based liposomes. These PE-based liposomes are unstable in buffer but quite stable in plasma. Furthermore, cholesterol improves the stability of PC-based liposomes in plasma but shows little effect on the PE-based liposomes.

In order to examine if the incubation with plasma has stabilized the PE-based liposomes, liposomes were incubated with 50% plasma at 37 °C for 1 h, separated from the plasma

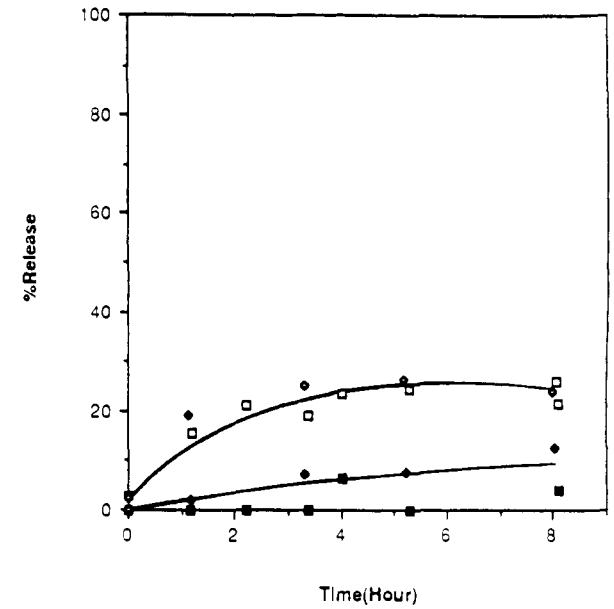

FIGURE 3: Stability of plasma-treated PE-based liposomes in 90% plasma or PBS at 37 °C. (□) DOPE/OA (2:1) in PBS; (◇) DOPE/OA/chol (2:1:2) in PBS; (□) DOPE/OA (2:1) in plasma; (◆) DOPE/OA/chol (2:1:2) in plasma.

components by chromatography on a Bio-Gel A-0.5M column (see Experimental Procedures), and retested for calcein release in PBS or in 90% plasma at 37 °C. As can be seen in Figure 3, the plasma-treated liposomes now became quite stable in PBS; only about 20% calcein release was observed after 8 h. Subsequent incubation in 90% plasma did not cause any significant calcein release. Again, cholesterol-free and cholesterol-rich liposomes behaved identically. Thus, preincubation of the PE-based liposomes in plasma had rendered the liposome stable. In fact, the stabilized liposomes could be stored at 4 °C for a few months without leakage of calcein (data not shown).

Eight different batches of human plasma taken from different individuals gave the same stabilization effect. Fresh or frozen plasma also showed the same activity. The activity was also seen in serum, whether dialyzed against PBS or not, indicating that the activity resides in some macromolecular

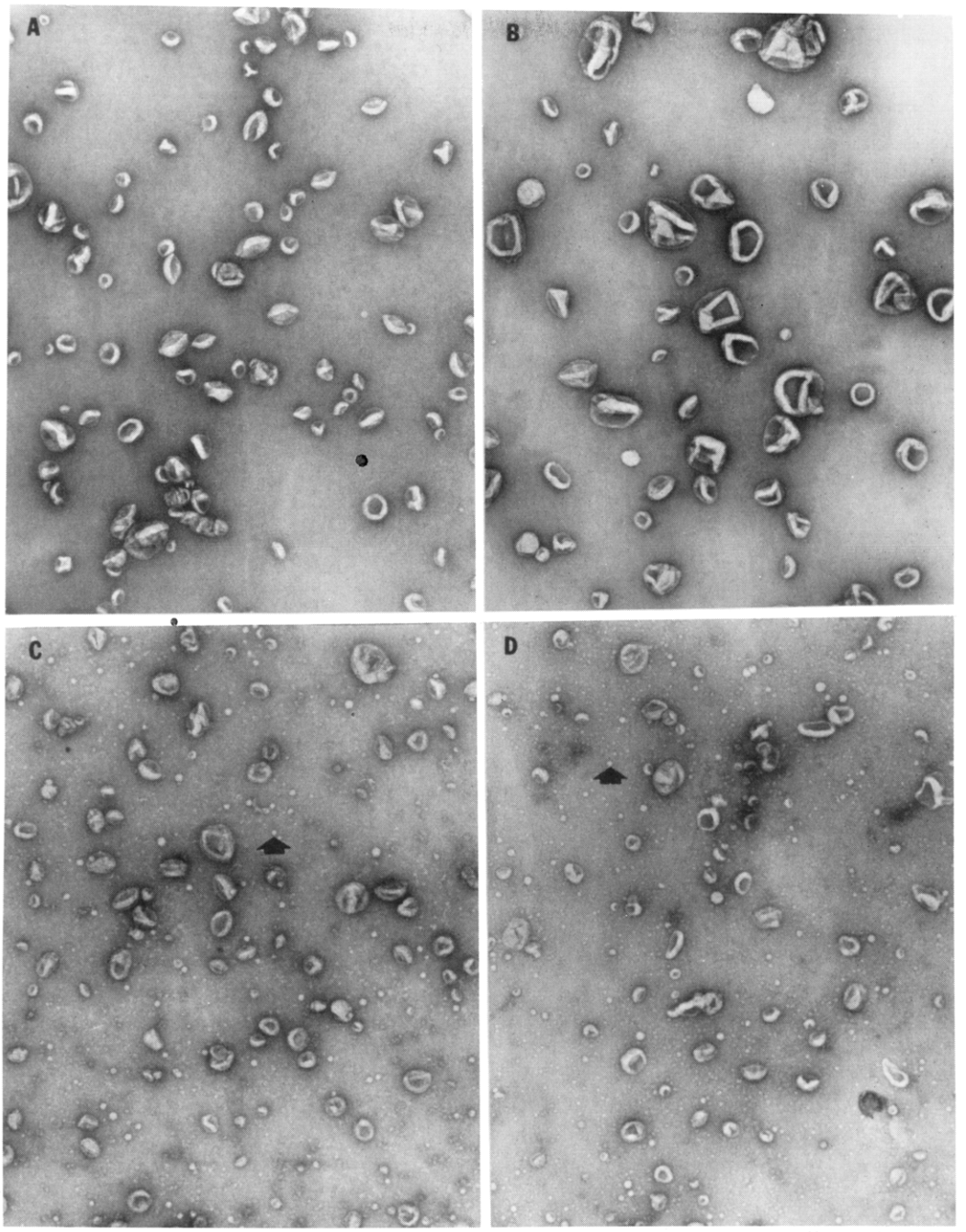

FIGURE 4: Electron micrographs of DOPE/OA (2:1) liposomes. The samples were negatively stained with 1% uranyl acetate. Magnification 24000×. The arrow shows lipidic particles. (A) Freshly sonicated liposomes mixed with an equal volume of PBS; (B) liposomes incubated in PBS for 24 h at 37 °C; (C) freshly sonicated liposomes mixed with an equal volume of plasma; (D) liposomes incubated in 50% plasma for 24 h at 37 °C.

component(s) common in the serum and the plasma.

Plasma Blocks the Aggregation and Fusion of the DOPE/OA Liposomes. When the DOPE/OA (2:1) liposomes were freshly sonicated, they appeared to be small unilamellar vesicles as revealed by negative-stain electron microscopy (Figure 4A). The average diameter of the liposome was 91 ± 25 nm (n = 232). Upon incubation at 37 °C in PBS, the liposomes began to fuse (Figure 4B) with an increase in the average diameter to 128 ● 36 nm (n = 216) after 24 h of incubation. The progress of liposome aggregation and fusion was also followed by dynamic laser light scattering. The average particle diameter grew from approximately 88 nm immediately after sonication to 128 nm during 24 h of incubation in PBS at room temperature. Thus, the destabilization of the small sonicated liposomes was accompanied by liposome fusion. However, this process could be completely blocked by adding human plasma to the liposome suspension. Figure 4C shows the morphology of the plasma-treated, freshly prepared liposomes, reisolated by gel filtration. Note that the liposomes were still unilamellar and nonaggregated. The average diameter was 79 ± 25 nm (n = 148), very close to that of the freshly sonicated liposomes without plasma treatment. The average diameter of the liposome (d = 78 ± 26 nm, n = 197) did not change after 24-h incubation in plasma at 37 °C (Figure 4D). The inhibitory effect of plasma on the aggregation and fusion of the liposomes could also be revealed by the light-scattering measurements. If plasma was added immediately after the sonication, the average particle diameter did not change with time.

Effect of Plasma Treatment on Albumin-Induced Liposome Lysis. Liposomes composed of unsaturated PE and OA are not stable in albumin solutions (Leventis et al., 1987). This

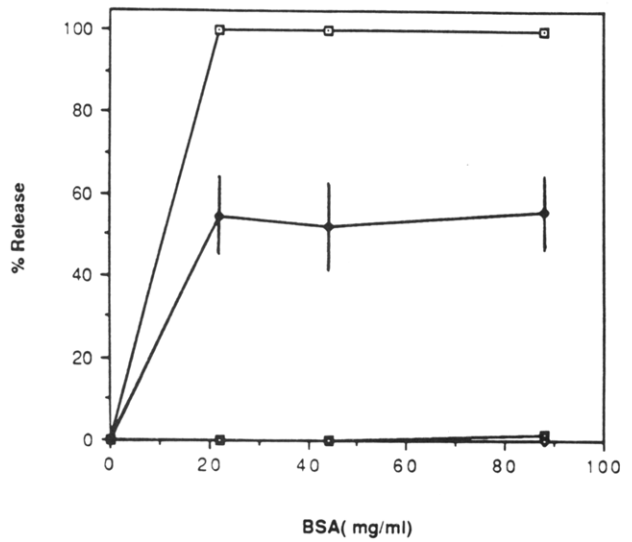

FIGURE 5: BSA-induced leakage of DOPE/OA (2:1) liposomes. (⊡) Untreated; (♦) 10% plasma treated; (□) 25% plasma treated; (◊) 50% plasma treated.

is probably due to the extraction of OA from liposome by albumin. We have investigated if the plasma-stabilized liposomes could be lysed by albumin (Figure 5). Liposomes not treated with plasma were very sensitive to BSA; complete lysis was observed at BSA concentration of 22 mg/mL or higher. However, if liposomes were pretreated with plasma, liposome lysis was completely inhibited. Complete protection of liposome by plasma was observed at a plasma concentration of 25% or above; 10% plasma only gave partial protection. These results imply that the stabilization of liposome by plasma is a stoichiometric reaction; i.e., the stabilization factor(s) in the plasma can be titrated.

Proteins Associated with the Plasma-Stabilized Liposomes. As part of the chemical characterization of the plasma-stabilized liposomes, liposomes were twice floated on a discontinuous Ficoll gradient after the incubation with plasma, and the proteins associated with the liposomes were analyzed by SDS–PAGE under reducing conditions (Figure 6). There were seven major polypeptides associated with DOPE/OA liposomes, four of them having molecular weights of 237K, 81K, 73K, and 66K. the molecular weights of the last three could not be accurately determined with the gel conditions used. Similar polypeptide patterns were found for other liposome compositions, i.e., DOPE/OA/chol (2:1:2); egg PC; egg PC/chol (6:4); DOPC/OA/chol (2:1:2). Thus, these major bands, although associated with the liposomes, are not the likely candidates for the plasma factor responsible for the stabilization of the DOPE/OA liposomes. One possible exception is a polypeptide with an electrophoretic mobility close to that of the ovalbumin used in the molecular weight standards; i.e., the molecular weight approximately equals 42.7K. This band was uniquely associated with the DOPE/OA liposomes. However, since it was absent in the proteins associated with the DOPE/OA/chol liposomes which are also stabilizable by plasma, the role of this polypeptide in the stabilization of the PE-based liposome could not be ascertained. Furthermore, all of the above-named large polypeptides associated with the DOPE/OA liposomes could be removed with trypsin digestion, yet the liposomes still retained calcein (data not shown). Therefore, it is not likely that these polypeptides are responsible for the liposome stabilization. These results, however, do not rule out the possibility that some smaller polypeptide(s) (M_r <40K) is (are) the liposome stabilizer. The electrophoretic conditions used in this study were not suitable

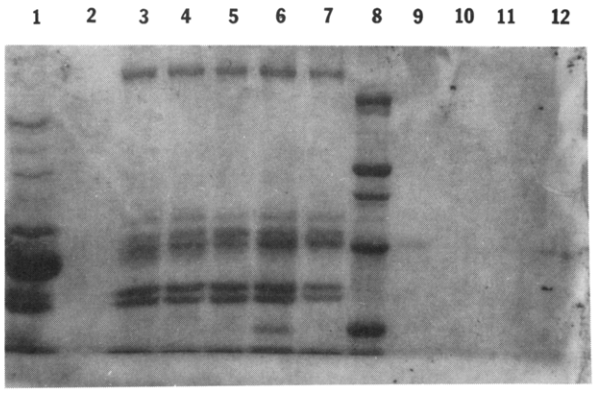

FIGURE 6: Plasma proteins associated with the plasma-treated liposomes. Liposomes were incubated with either PBS or plasma for 1 h at 37 °C. The liposomes were then floated in a discontinuous Ficoll gradient (see text for details) and analyzed on a 7.5% polyacrylamide gel in the presence of SDS and mercaptoethanol. (1) Whole human plasma; (2) blank; (3) egg PC liposomes treated with plasma; (4) egg PC/chol (6:4) liposomes treated with plasma; (5) DOPC/OA/chol (2:1:2) liposomes treated with plasma; (6) DOPE/OA (2:1) liposomes treated with plasma; (7) DOPE/OA/chol (2:1:2) liposomes treated with plasma; (8) molecular weight standards (myosin, β-galactosidase, phosphorylase B, BSA, ovalbumin); (9) whole human plasma floated in the absence of liposomes; (10) egg PC liposomes treated with PBS; (11) DOPC/OA/chol (2:1:2) liposomes treated with PBS; (12) DOPE/OA/chol (2:1:2) liposomes treated with PBS.

to detect low molecular weight proteins.

Lipids Associated with the Plasma-Stabilized Liposomes. We first checked if OA had been extracted away from the DOPE/OA liposomes by using [^{14}C]OA as a marker. After incubation with the plasma, liposomes were floated on a Ficoll gradient, and the distribution of [^{14}C]OA in the gradient was determined. Approximately 28 ± 5.0% (n = 5) of the total [^{14}C]OA was found in fractions at the top portion of the gradient. The top fractions contained most of the liposomes as judged by the turbidity and quenched calcein fluorescence of the liposomes. These results indicate that approximately 72% of the original OA was extracted from the liposomes during the incubation with the plasma. The inclusion of cholesterol did not block the OA extraction.

The lipids of the plasma-treated liposomes, original composition DOPE/OA (2:1), were extracted and separated by thin-layer chromatography on silica gel. Figure 7 shows the chromatogram. Apparently, the lipid composition of the plasma-treated liposomes was more complex than the original composition. The relative abundance of the lipids in the treated liposomes, as measured by the intensity of the iodine vapor stain and by charring the TLC plate after spraying with sulfuric acid, was in the order of PE > PC ≥ chol > lysoPC > fatty acids. Although the lipids were not quantitated, the protein:lipid ratio of the plasma-treated liposome was determined to be 0.51 ± 0.02 (w/w) (n = 6). Thus, the plasma-treated liposomes contained about one-third protein and two-thirds lipid by weight. Thus, they were "proteoliposomes".

pH Sensitivity of the Plasma-Treated Liposomes. The DOPE/OA (2:1) liposomes rapidly destabilize in a weakly acidic environment (pH <7) (Straubinger et al., 1985; Wang et al., 1986). Since the composition of the liposome had changed significantly upon treatment with plasma, one would expect that the pH sensitivity of the liposome would also be modified. This was determined directly, and the results are shown in Figure 8. Quite obviously, the plasma-treated liposomes, whether originally cholesterol-free or -rich, had become insensitive to acid. Only 10–20% calcein release was observed at pH 4, as compared with 80–95% release at the same pH for the liposomes without the plasma treatment.

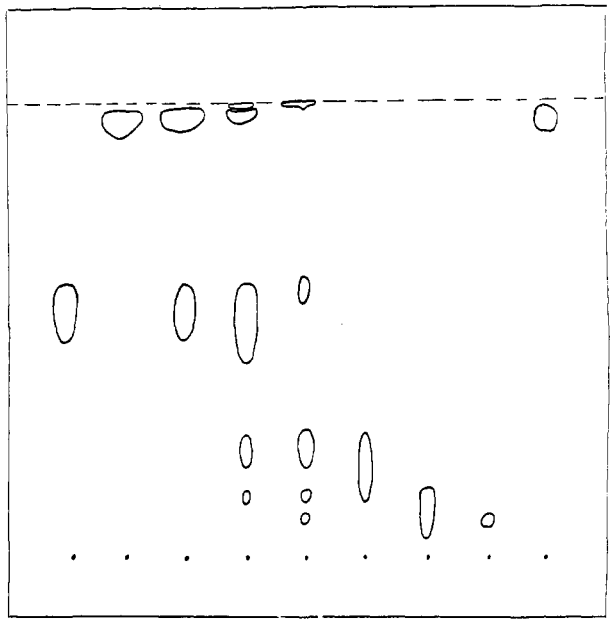

FIGURE 7: Thin-layer chromatography of lipids extracted from DOPE/OA liposomes (100 μg of lipids per lane). Solvent contains $CHCl_3$/acetone/methanol/acetic acid/H_2O (6:8:2:2:1). From left to right: DOPE; OA; liposome treated with PBS; liposomes treated with plasma; lipids extracted from plasma; egg PC; lysoPC; sphingomyoline; cholesterol.

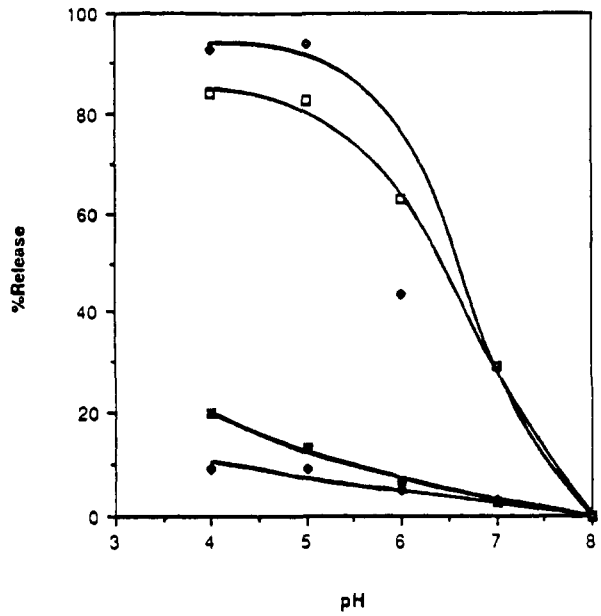

FIGURE 8: Acid-induced leakage of PE-based liposomes. (□) DOPE/OA (2:1) and (◇) DOPE/OA/chol (2:1:2) treated with PBS; (■) DOPE/OA (2:1) and (◆) DOPE/OA/chol (2:1:2) treated with 50% plasma.

Effect of Liposome Size on the Plasma Stabilization of Liposomes. We have previously reported that large unilamellar liposomes composed of DOPE/OA (2:1) are not stable in human plasma unless cholesterol is included in the lipid composition (Liu & Huang, 1989). This report, seemingly conflicting with the results described here, could be explained on the basis of the liposome size. The liposomes used in previous studies were prepared by a detergent dialysis protocol. The present liposomes were prepared by sonication, resulting in much smaller liposomes. In order to test this hypothesis, a systematic study on the effect of liposome size on the plasma stabilization of liposomes was undertaken. Liposomes were prepared with both methods, and the average particle size was

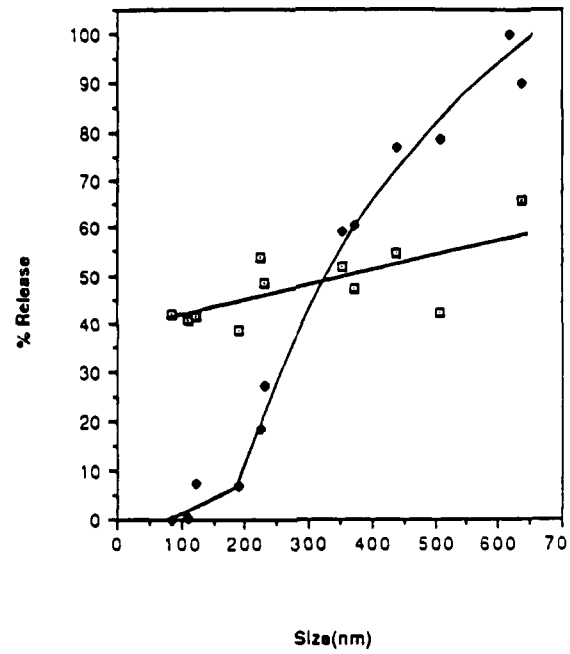

FIGURE 9: Size dependence of liposome stability in plasma. Percent calcein release of DOPE/OA liposomes (50–100 nmol of lipid) with various diameters after 4-h incubation in 90% plasma (◆) or in PBS (□).

determined by laser light scattering. To obtain intermediate-sized liposomes, liposomes prepared by detergent dialysis method were extruded through polycarbonate filters of defined pore size, and the average particle diameter of the extruded liposomes was also determined by laser light scattering. These liposomes were then gel filtered to remove the free, unentrapped calcein and tested for dye release after 4-h incubation at 37 °C in PBS or in 90% human plasma. The result is shown in Figure 9. The stability of the liposomes in PBS was not very dependent on the liposome size; larger liposomes released only slightly more calcein than the smaller ones. However, the dye release from liposomes incubated in the plasma was a sensitive function of the liposome size. Small liposomes ($d \lesssim 200$ nm) released less than 10% calcein in 4 h, whereas larger liposomes ($d > 600$ nm) released almost all of the entrapped dye during the same period of incubation. Consistent with previous studies (Liu & Huang, 1989), large liposomes were more stable in PBS than in plasma. Small liposomes showed the opposite behavior; i.e., they were more stable in plasma than in PBS. Thus, liposome size determines the extent of liposome stabilization; only small liposomes can be stabilized by the plasma factor(s).

Discussion

The results presented here clearly indicate that small liposomes composed of DOPE and OA, but not those composed of DOPC and OA, are stabilized by some factor(s) in the human plasma. This conclusion is supported by several experimental results. First, only DOPE-based liposomes are stable in the plasma or serum, but not the DOPC-based liposomes unless they contain cholesterol (Figures 1 and 2). DOPE-based liposomes can be isolated and appear to be stable upon subsequent incubation with PBS or albumin (Figures 3 and 5), or upon prolonged storage. Finally, the spontaneous aggregation and fusion of the DOPE/OA liposomes are completely blocked by incubation with plasma (Figure 4). These results suggest that some factors in the human plasma have transferred to the liposome membrane, giving rise to the stabilization effect.

An important observation is the effect of liposome size on the plasma stabilization. Only small liposomes with diameters equal to or smaller than approximately 200 nm are stabilized; liposomes with greater diameters are less effectively stabilized (Figure 9). This result is quite understandable in view of the fact that hydrophobic molecules insert themselves into the membranes of the small liposomes with small radius of curvature more effectively than those of the larger liposomes with large radius of curvature (MacDonald & MacDonald, 1988). Insertion of an amphipathic protein such as the cytochrome b_5 also prefers small liposomes over larger ones by a factor of at least 20 (Greenhut & Roseman, 1985). This is due to the fact that high curvature of the smaller liposome gives rise to a lower surface pressure than the larger liposome (MacDonald, 1988; Schindler, 1980). The surface pressure of a 20-nm PC liposome is 15 dyn/cm, which progressively increases to 46 dyn/cm for a 200-nm liposome (MacDonald, 1988). Notice that the surface pressure cited here refers to that of the outer monolayer of the unilamellar liposome. Lipids located in the inner monolayer of a small liposome are likely packed much tighter, thus giving rise to a higher surface pressure. The transmembrane gradient of the surface pressure diminishes with increasing diameter of the liposome. This is the reason why small unilamellar liposomes, under appropriate conditions, spontaneously aggregate and fuse with each other to become larger liposomes (Wong et al., 1982).

The transmembrane gradient of the surface pressure for the small liposomes composed of DOPE/OA is likely to be greater than that of the same size liposomes composed of PC or PC/OA. This is because the head group of PE hydrates poorly as compared to the head group of PC (Cullis & de Kruijff, 1979). Furthermore, the bulkiness of the unsaturated acyl chains of DOPE prevents a close van der Waals interaction of the hydrocarbon chains. The combined effect of the head group and acyl chains has been described by a "molecular shape" theory, assigning the shape of an unsaturated PE being that of an inverted cone, i.e., a small head and a bulky tail (Cullis & de Kruijff, 1979). Amphiphiles of the inverted cone shape favor the packing of the hexagonal H_{II} phase, unless complemented by other amphiphiles of the opposite shape, i.e., molecules which contain a head group with relatively good hydration and saturated acyl chains. Thus, DOPE is better suited to locate in the inner monolayer of a small liposome, because it closely resembles the packing of the hexagonal H_{II} phase, giving rise to a relatively high surface pressure at the inner monolayer. On the other hand, DOPE on the outer monolayer of a small liposome does not pack well at all, giving rise to a relatively low surface pressure. In fact, the distribution of OA is likely asymmetric; more OA should be found in the outer monolayer for the sake of compensation with DOPE than in the inner monolayer. Such a predicted transmembrane gradient in surface pressure could be the driving force for the spontaneous aggregation and fusion of the small DOPE/OA liposomes at 37 °C (Figure 4), leading to the instability of the liposomes in PBS (Figure 1). Removal of OA from the small DOPE/OA liposomes would result in a rapid destabilization of the liposome. Indeed, this was observed when the liposomes were incubated with BSA (Figure 5).

Removal of the majority of the OA from the DOPE/OA liposomes was also observed when the liposomes were incubated with human plasma, but the liposomes were not destabilized. Instead, stabilization of the liposomes had occurred. We proposed that some plasma factor(s), lipid and/or protein, must be rapidly inserted into the outer monolayer of the small liposome, thereby increasing the surface pressure of the outer monolayer and stabilizing the entire liposome structure. Insertion of the plasma factor(s) probably does not, or to a much lesser extent, involve the inner monolayer of the liposome, because the surface pressure of the inner monolayer should be already high. The surface pressure of the outer monolayer of a larger liposome is considerably higher than that of a small liposome (MacDonald, 1988). Insertion of stabilizing factor(s) from plasma into the large liposomes is less likely. In the event of insertion, it probably inserts all the way to the inner monolayer and leads to liposome leakage, because the surface pressure of the inner monolayer of a larger liposome is about the same as that of the outer monolayer. Furthermore, the model also predicts that small liposomes composed of mainly PC are not stabilized by the same mechanism. Again, the outer monolayer of PC-based liposome exhibits higher surface pressure than the PE-based liposome and is thus less accommodative to the stabilization factor(s) from the plasma. Destabilization of PC-based liposome is also due to the fact that PC is transferred from liposomes to HDL particles (Tall & Green, 1981; Kirby et al., 1980; Senior et al., 1983).

While the evidence for the action of the stabilization factor(s) is strong, the molecular identity of the factor(s) is not clear. A number of possibilities exist upon examining the composition of the stabilized liposomes. Among the lipids found in the stabilized liposomes, lysoPC is probably the one which has the highest complementarity activity for the bilayer packing of DOPE. This is because lysoPC contains a phosphorylcholine head groups which hydrates well and a single, nonbulky fatty acyl chain which interacts well with other acyl chains; i.e., it is an amphiphile with the shape of that of a cone (Cullis & de Kruijff, 1979). Mixtures of lysoPC and unsaturated PE form a stable bilayer phase (Madden & Cullis, 1982). However, lysoPC is also a lipid which binds strongly with albumin (Klopfenstein, 1969). The fact that the stabilized liposomes stay stable upon subsequent incubation with the fresh plasma (Figure 3) indicates that the stabilization component(s) in the stabilized liposome is (are) not removed by the fresh plasma. This is unlikely the case if lysoPC were the stabilization factor. It is possible that the stabilization is brought about by a minor lipid which escaped from our detection method. Gangliosides would be a good candidate. Among the major polypeptide bands shown on SDS–PAGE (Figure 6), none of them is a likely candidate for the stabilization factor. This is because many of them also associate with the DOPC/OA liposomes which are not stabilized. However, our gel electrophoresis condition does not separate the small molecular weight polypeptides well. It is possible that one or more of the smaller blood proteins could serve as the stabilization factor. This is particularly interesting because the apolipoproteins from HDL are known to incorporate into liposome membranes (Luke et al., 1980; Williams & Scanu, 1986), and all of the HDL apolipoproteins have molecular weights smaller than 30K. Apolipoprotein A_1, in particular, readily inserts into the liposome membrane (Luke et al., 1980; Williams & Scanu, 1986; Ibdah & Phillips, 1988). Identification of the stabilizer(s) is currently under way in our laboratory.

While the stabilization factor(s) remain(s) unknown, the plasma-stabilized small unilamellar liposomes represent a new class of liposomes which show a superb stability both for long-term storage and during the incubation with blood. The biodistribution of the stabilized liposome is currently under study. It will be interesting to see if such small liposomes containing a high level of protein stay in the circulation for a relatively long period of time after intravenous injection. It

is known that small liposomes have a longer circulation half-life than the larger ones (Juliano & Stamp, 1975). The drug carrier potential of the stabilized liposome also awaits further exploration.

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