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PHASE TRANSITION RELEASE, A NEW APPROACH TO THE INTERACTION OF PROTEINS WITH LIPID VESICLES

APPLICATION TO LIPOPROTEINS

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To study the interaction of proteins with lipid bilayers, we have developed a new experimental approach based on the release of a water-soluble fluorescent dye from liposomes during scans through the lipid phase transition temperature. The fluorescence of carboxyfluorescein is quenched at high dye concentrations inside the vesicles but appears when the dye is released and diluted into the external medium. This new approach, phase transition release, is here applied to the interaction of serum lipoproteins and apolipoproteins with liposomes (for the most part, small unilamellar vesicles of dipalmitoylphosphatidylcholine). The major findings are these: (i) All of the lipoproteins and apolipoproteins tested induce a smooth, rapid release of carboxyfluorescein, essentially complete within a few seconds. HDL apolipoprotein induces 50% carboxyfluorescein release at a lipid/protein molar ratio of 3 400 : 1, whereas a ratio of 160 : 1 is required for native HDL. (ii) The interaction is all-or-none and irreversible. It involves a sufficient perturbation of bilayer structure to permit equal release of carboxyfluorescein (M_r 373) and inulin (M_r 5 500). In the case of HDL apolipoprotein, this release accompanies formation of a relatively homogeneous population of vesicular recombinant structures. Only at much higher protein/lipid ratios are the often-studied small, disc-shaped recombinants formed. (iii) Much more dye is released if the transition temperature is approached from below than if it is approached from above. (iv) Phase transition release is seen with multilamellar and reverse phase evaporation vesicles, though with patterns different from those seen with small unilamellar vesicles. (v) A large number of proteins are found not to induce phase transition release, even at concentrations of at least 1000-times those required for the HDL apolipoprotein effect. These include trypsin, chymotrypsin, pronase, bovine serum albumin (crystalline), ovalbumin, rabbit immunoglobulin G (and its $F(ab)'_2$ and F_c fragments), rabbit immunoglobulin M, hemoglobin, hen lysozyme, synexin, ankyrin, myosin, and microtubule-associated proteins. Tubulin and actin, on the other hand, do induce phase transition release. In addition to its use for analysis of protein-bilayer interaction, phase transition release provides a way of reconstituting relatively water-soluble proteins into vesicles, under quantitative control and without detergent.

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Abbreviations: DPPC, dipalmitoylphosphatidylcholine; DSPC, distearoylphosphatidylcholine; $F(ab')_2$ and F_c, fragments of immunoglobulin G; HDL, LDL, IDL, VLDL: high density, low density, intermediate density, and very low density lipoproteins; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; NaKCl-H, 139 mM NaCl/6 mM KCl, buffered to pH 7.4 with 10 mM Hepes; T_m, endothermic phase transition temperature ('melting' temperature) of the lipid.

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Introduction

Lipid-protein interactions are central to the integrity and function of numerous biological structures, among them the serum lipoproteins and cell membranes. We present here a new approach, which we call 'phase transition release', to the study of such interactions as they occur at the liquid-crystalline phase transition temperature (T_m) of lipid bilayers [1–4]. In brief, we encapsulate a small hydrophilic solute in lipid vesicles and monitor its release – as an index of interaction of the vesicle with protein in the medium – while the temperature is scanned through T_m. In principle this could be done in a variety of ways, for example by following an enzymatic reaction attending release of solute into the space outside of the vesicles. Our current technique, however, is based on self-quenching of fluorescence. When the water-soluble fluorophore carboxyfluorescein is encapsulated in lipid vesicles at sufficiently high concentration (e.g., 100 mM), its fluorescence is almost completely quenched by interaction between neighboring fluorophore molecules [5,6]. Release of the carboxyfluorescein from vesicles can therefore be equated directly with an increase in fluorescence as the temperature is scanned through T_m. Multilamellar vesicles are well known to be maximally permeable and susceptible to osmotic stresses near T_m [7–13]. Small unilamellar vesicles are more stable in that temperature range, unless a phase transition release-promoting protein is present.

We will consider here the interaction of lipid vesicles with serum lipoproteins and apolipoproteins. Numerous studies over the last decade have shown these proteins to have detergent-like action leading to the breakdown of phospholipid vesicles and formation of specific lipid-protein recombinant structures [14–29]. However, the molecular details of protein insertion and its sequelae are poorly understood. Here we describe the use of phase transition release to delineate some of the issues of mechanism. Elsewhere we develop specific models of the interaction with vesicles of one particular apolipoprotein, AI (unpublished results).

Phase transition release was developed in our laboratory to assay such interactions between liposomes and serum components, but we have also used it with a variety of other proteins, including tubulin [30], actin, myelin basic protein and a hepatic receptor for asialoglycoproteins [31]. Phase transition release provides a very quick assay for interaction at T_m, and gives considerable detailed information on the mechanism by which recombination or insertion takes place. We use it in combination with other biochemical, spectroscopic and morphological methods to develop a profile of the interaction and of its consequences for the lipid and for the protein.

Materials and Methods

Liposomes. Dipalmitoylphosphatidylcholine (DPPC) and distearoylphosphatidylcholine (DSPC) were obtained from Avanti Biochemical Co. (Birmingham, AL). Samples of 100 μg gave single spots on thin-layer chromatograms (silica gel G; developed with $CHCl_3/CH_3OH/H_2O$, 64 : 24 : 4; visualized with I_2 vapor). The phase transition temperatures by differential scanning calorimetry were 42°C for multilayers of the DPPC and 54°C for multilayers of the DSPC. 5(6)-Carboxyfluorescein (Eastman Kodak, Rochester, NY) * was treated with activated charcoal and precipitated from approx. 1 : 2 ethanol/water (unpublished method of Hagins, W.A. and Yoshikami, S.). At least one recent lot of carboxyfluorescein from Eastman Kodak (lot No. CX4) contained troublesome hydrophobic impurities not removed by the above protocol. At the suggestion of Dr. R. Haugland, we eliminated these impurities by elution with distilled water on a 2.6 × 30 cm column of Sephadex LH20 (Pharmacia, Piscataway, NJ). A 40-ml sample of 200 mM aqueous carboxyfluorescein at pH 7.4 could be eluted on such a column with efficient separation (unpublished data).

The lipids were dried from benzene onto a glass vial under a stream of argon, and lyophilized overnight. We used either 25 mg DPPC plus 12 mg DSPC

* There has been some confusion as to the correct chemical name for carboxyfluorescein. Originally sold as '6-carboxyfluorescein' (Kodak catalog No. 49), the dye was renamed '4(5) carboxyfluorescein' (Kodak catalog No. 50) after it was realized that the preparation was, in fact, a mixture of two isomers. The correct name appears to be '5(6)-carboxyfluorescein'. In the Chemical Abstracts the 6- isomer of the closed, lactone form is listed as 3′,6′-dihydroxy-3-oxospiro(isobenzofuren-1(3H),9′-(9H)xanthen)-6-carboxylic acid (registry No. [3 301–79-9]).

(for 7 : 3 mol/mol DPPC/DSPC vesicles) or 25 mg DPPC. DPPC and DPPC/DSPC gave qualitatively and quantitatively similar results in all phase transition release studies except that T_m was several degrees higher for the mixture, as expected. Carboxyfluorescein solutions (100 mM) were made up in distilled water by titration to pH 7.4 with 10 M NaOH. A 5-ml portion of the solution was warmed in a water bath to 50°C and added to the vial, also at 50°C. The suspension was hydrated, with repeated vortex-mixing, at the same temperature for about 15 min. For hand-shaken multilamellar vesicles, 0.5 ml of the suspension was allowed to cool to room temperature and passed over a column of Sephadex G-25 (Pharmacia). The rest of the suspension was sonicated to form small unilamellar vesicles at 50°C under argon for 1 h with a titanium microprobe (Heat-Systems sonicator, model W350, Plainview, NY) operating at power level 4. Suspensions clarified within 5 min. After sonication, the suspension was maintained above T_m for 15 min and centrifuged briefly at low speed to remove titanium particles. 3 ml of the suspension was then eluted with NaKCl-H solution (139 mM NaCl/6 mM KCl/10 mM Hepes, pH 7.4) on a 1.6 × 30 cm column of Sepharose 4B (Pharmacia) at room temperature. If the sonicate became turbid before chromatography, good small unilamellar vesicles were not obtained. There is clearly still 'magic' to the preparation of relatively stable small unilamellar vesicles of DPPC. Sometimes the vesicles became turbid or changed phase transition release characteristics within hours (cf. Ref. 32). At other times they remained stable in all properties for a month. One possible variable that we have not investigated is the presence of a 1,3-diacyl isomer of the usual 1,2-diacylphosphatidylcholine [33]. T_m for the small unilamellar DPPC vesicles was 37–38°C, as determined from depolarization of the fluorescence of 1,6-diphenyl-1,3,5-hexatriene [30]. Probably because of stresses arising from the low radius of curvature, this transition temperature is several degrees lower than that of multilamellar vesicles made with the same lipids.

Large unilamellar vesicles were prepared by the reverse phase evaporation method of Szoka and Papahadjopoulos [34], using 25 mg DPPC in 1 ml 70 mM carboxyfluorescein solution. After removal of most of the organic solvent (3 ml isopropyl ether/1 ml chloroform) in a rotary evaporator at 45°C, an additional 1 ml carboxyfluorescein solution was added to aid in further removal of solvent. The suspension was then passed through an 0.2 μm polycarbonate filter (Nuclepore Corp., Pleasanton, CA) [35], and an 0.5-ml portion was eluted with NaKCl-H on a Sephadex G-25 column (Pharmacia) to remove free carboxyfluorescein. Similarly prepared reverse phase evaporation vesicles appeared mostly unilamellar by two criteria: (i) approx. 50% of the ^{31}P-NMR signal from the lipid head groups of intact vesicles could be abolished by the line-broadening agent Mn^{2+} (method based on Ref. 36); (ii) when 2% phosphatidylethanolamine (Avanti) was included in the lipid, approx. 50% of the free amino groups could be modified by incubation of intact vesicles with trinitrobenzene sulfonate, whereas all could be modified if the vesicles were first disrupted by detergent (method based on Ref. 37).

Isolation and characterization of lipoproteins. Human lipoproteins were isolated by sequential ultracentrifugation from the plasma of a fasted healthy male subject. Very low density lipoproteins (VLDL) and intermediate density lipoproteins (IDL) were isolated in a 60 Ti rotor (Beckman Instruments) by centrifugation at 50 000 rev./min for 16 h at d = 1.006 and d = 1.02, respectively. Low density lipoproteins (LDL; d = 1.02–1.063 or 1.02–1.05) were obtained by centrifugation at 59 000 rev./min in a 60 Ti rotor for 18 h. The d = 1.02–1.05 LDL was washed at d = 1.05 by centrifugation for 16 h at 59 000 rev./min. HDL (d = 1.063–1.21) or HDL_3 (d = 1.125–1.21) was isolated by centrifugation for 42–48 h at 59 000 rev./min and washed by a second centrifugation at d = 1.21 for 24 h. HDL_3 is a subfraction of HDL enriched in apolipoprotein A1 with respect to the other apolipoproteins. It behaved identically with HDL apolipoprotein (and with apolipoprotein A1 as well) in phase transition release experiments. For present purposes, it could be used interchangeably with HDL apolipoprotein. The isolated lipoproteins were extensively dialyzed against 0.15 M NaCl/0.01% EDTA, pH 7.4 and concentrated using an Amicon filtration device. SDS-polyacrylamide gel electrophoresis [38] indicated normal lipoproteins. Protein (measured by the method of Lowry et al.), triacylglycerol, phospholipid and cholesterol contents of the lipoprotein fractions were similar to those reported in the literature (see Ref. 39). To obtain

'HDL apolipoprotein' the isolated HDL was dialyzed against 0.1% EDTA, lyophilized and extracted with cold chloroform/methanol (2 : 1, v/v). As determined by polyacrylamide gel electrophoresis, the apolipoprotein composition of the delipidated lipoproteins was unchanged from that of the native HDL.

Phase transition release measurements. Liposome suspensions were routinely assayed for fluorescence before and after addition of Triton X-100 to a final concentration of 0.4%, as described previously [40, 41]. On the basis of the post-Triton carboxyfluorescein values or incorporated [^{14}C]DPPC, they were diluted to approx. 30–60 μM lipid for use in phase transition release.

The fluorometry system used for phase transition release is diagrammed schematically in Fig. 1. The sample chamber of an Aminco-Bowman fluorometer (American Instrument Co., Silver Spring, MD) was heated to 47°C by a circulating water bath. The sample was placed in a small rectangular quartz cuvette (3 X 3 mm internal diameter, 5 X 5 mm outer diameter, 24 mm high, Precision Cells, Hicksville, NY), and the cuvette was seated in an adaptor (No. J-6114, American Instrument Co.) at room temperature. The cuvette and adaptor were then transferred

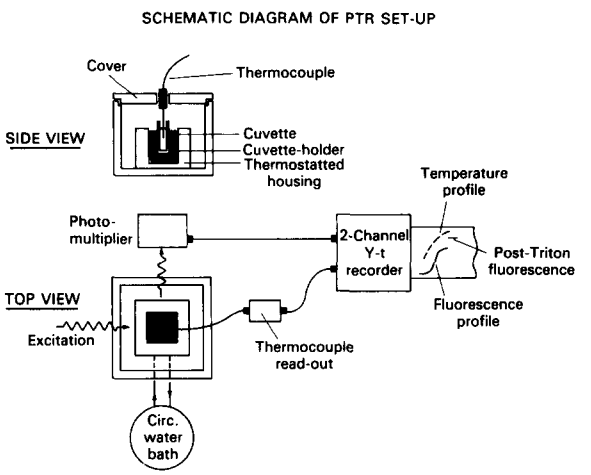

Fig. 1. Schematic diagram of the experimental apparatus for phase transition release. The suspension of protein and vesicles is placed in a small cuvette and heated rapidly through T_m, as described in the text. Temperature and fluorescence profiles are monitored continuously on a two-channel y-T recorder. PTR, phase transition release.

to the heated sample chamber, and a copper-constantan thermocouple probe (Type PT-6, Bailey Instruments Inc., Saddle Brook, NJ) was inserted to lie 1 mm above the lightpath. For reliable temperature determination, it was important that the probe tip be fully submerged in the sample. Fluorescence was monitored continuously on a strip-chart recorder, with excitation at 470 nm and emission at 515 mm. Unless otherwise noted, heating rates at passage through T_m were 10–15°C/min. At these rates, the temperature readings were accurate to within 1°C. For routine quantitation of release, we often omitted the thermocouple probe.

Lipoprotein solutions were serially diluted with NaKCl-H in a microtiter plate kept on ice. A 135-μl portion of vesicle suspension was mixed with 15 μl of the appropriate dilution of lipoprotein, and 135 μl of the resulting mixture was pipetted into the cuvette for measurement. After the temperature scan, 5 μl Triton X-100 diluted 1 : 10 with distilled water was added to the cuvette with an Oxford 5-μl micropipette, the thin plastic tip of which was then removed from the pipette with forceps and used for mixing. Fluorescence and temperature were monitored on a two-channel y-T recorder (Heath-Schlumberger, model SR-206). It proved feasible to scan one sample approximately every 90 s. Unless otherwise noted, all of the experiments were run in NaKCl-H. However, for the lipoproteins the results were identical in Hank's balanced salt solution (which contains, among other additions, 2 mM Ca^{2+}). For most purposes, the fractional release of carboxyfluorescein in phase transition release could be estimated satisfactorily simply by inspection of scans such as those in Fig. 4. The details of calculations used to obtain Figs. 3 and 4, as well as specific stoichiometries, are presented in the Appendix.

For calculations of stoichiometry, we used molecular weights of 23 000 for HDL apolipoprotein and 733 for DPPC. We assumed 5 000 lipid molecules in a small unilamellar vesicle. The molecular weight of HDL apolipoprotein was calculated on the basis of a 4.4 : 2 : 1 molar ratio of apolipoprotein A1 (28 300) [42], apolipoprotein A2 (17 400) [42], and the apolipoproteins C (about 8000).

Comparison of carboxyfluorescein and inulin release. Small unilamellar vesicles were formed in 100 mM carboxyfluorescein and [^{3}H]inulin (0.14 mCi/

mg; New England Nuclear, Boston, MA). Encapsulation of the two solutes was commensurate. Portions (100 μl) of the suspension were passed through T_m in a cuvette, with or without HDL_3 apolipoprotein, and returned to room temperature. Of each portion, 80 μl was eluted with NaKCl-H on a Sephadex G-200 column previously saturated with DPPC. A portion of each fraction was read for fluorescence after addition of Triton, and a portion was counted for ^3H.

Results

Phase transition release can be used to identify a protein-bilayer interaction at T_m and then to address a series of questions about that interaction. We now show some of the types of information obtained in this way for whole HDL and for HDL apolipoprotein.

Phase transition release resulting from interaction of HDL with DPPC bilayers near T_m

Fig. 2 shows a series of fluorescence profiles indicating carboxyfluorescein release at different HDL concentrations. The downward slope seen in the control scan with carboxyfluorescein and at the ends of scans with free liposomes is a result of the temperature dependence of carboxyfluorescein fluorescence. The second scan in Fig. 2 indicates that with vesicles there was very little increase in fluorescence near T_m in the absence of HDL. With some other batches of small unilamellar vesicles, we saw essentially no transition release in the absence of protein, and the amount seen in Fig. 2 was attributable to contamination with larger, osmotically responsive forms (see later results). This spontaneous release could be eliminated by an initial cycle through T_m (and rechromatography on Sephadex if desired, to eliminate the released carboxyfluorescein). However, the lipid-protein interaction could be assessed perfectly well without such pretreatment of the vesicles. At concentrations greater than 20 μg HDL/ml, the release of dye was virtually complete. There was no discernible release of carboxyfluorescein at room temperature at any protein concentration, whereas release near T_m was smooth and rapid, taking at most a few seconds. To calculate a percentage release of carboxyfluorescein, it was necessary to make small corrections for dilution by Triton and for the temperature dependence of carboxyfluorescein fluorescence. Then by the calculation described in the Appendix, we obtained the profile shown in Fig. 3. The lipid/protein molar ratio sufficient for 50% protein-induced release was 160 : 1.

It should be emphasized that, on the time scale of these phase transition release scans, there was very little release from DPPC small unilamellar vesicles at

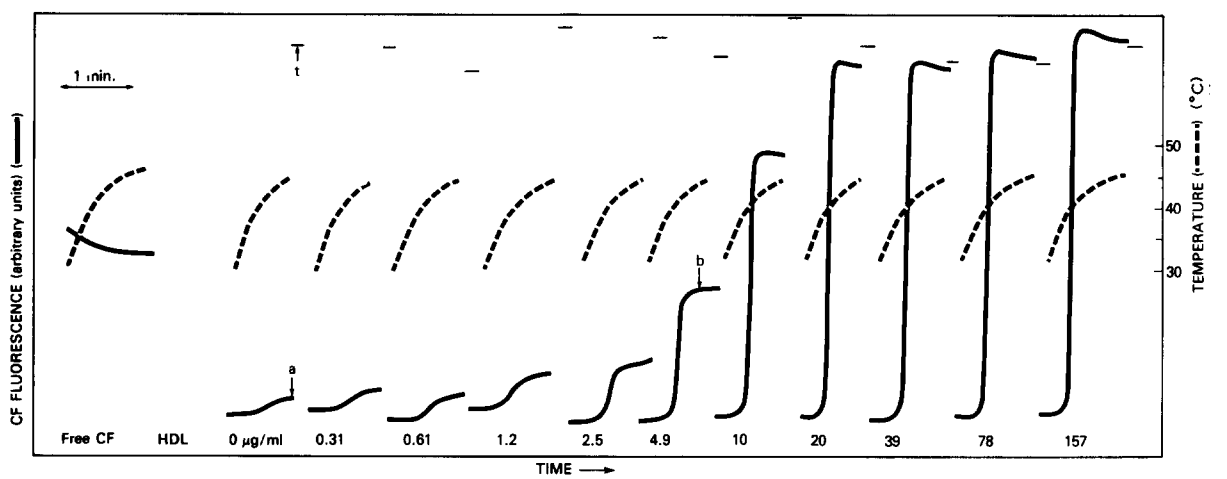

Fig. 2. Phase transition release profiles for small unilamellar DPPC/DSPC vesicles, as a function of HDL concentration. The letters a, b, and t indicate fluorescence values used in calculations described in the Appendix. (t) indicates 100% release (after addition of Triton, with a 4% correction for the volume of detergent added). The first profile is that for free carboxyfluorescein, indicating the temperature dependence of fluorescence. Subsequent profiles are for vesicles. The vesicle lipid concentration was 60 μM for each scan, and the HDL protein concentrations were varied as indicated at the bottom of the figure. CF, carboxyfluorescein.

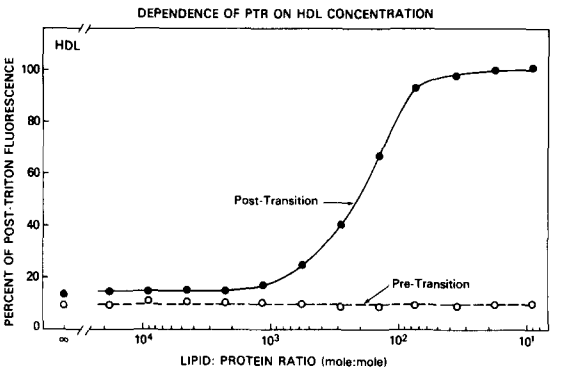

Fig. 3. Dependence of phase transition release (PTR) on HDL concentration, for small unilamellar DPPC/DSPC vesicles. The curves were calculated from Fig. 2, as described in the Appendix. At high protein concentrations, release approached 100%. The point of 50% release (see Appendix for calculation) was at a protein/lipid ratio of 160 : 1.

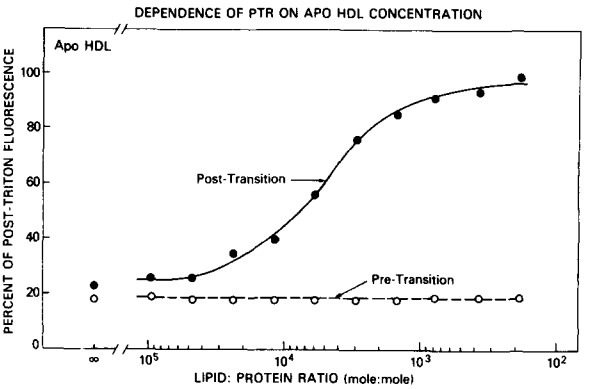

Fig. 4. Dependence of phase transition release (PTR) on HDL apolipoprotein (APO HDL) concentration, for small unilamellar DPPC/DSPC vesicles. The vesicle lipid concentration was 36 μM. The point of 50% release (see Appendix for calculation) was at a protein/lipid ratio of 3 400 : 1.

temperatures other than T_m. At 4°C, the time constant for release was on the order of weeks. Release was also slow for lipids such as dioleoylphosphatidylcholine (fluid) and DSPC (solid) with no transition in the temperature range scanned. For DPPC and DPPC/DSPC mixtures, experiments at a series of constant temperatures showed the previously described peak in release rate near T_m, and somewhat higher rates above than below the peak. HDL and HDL apolipoprotein greatly increased the rates near and above T_m.

Comparison of HDL and HDL apolipoprotein in phase transition release

Fig. 4 shows the titration profile of carboxyfluorescein release obtained with HDL apolipoprotein. The point of 50% release for HDL apolipoprotein corresponded to a lipid/protein molar ratio of 3 400 : 1. Comparison with Fig. 3 shows that HDL apolipoprotein effected the same degree of release at about a 20-fold lower protein concentration than did native HDL.

Irreversibility of the protein-vesicle interaction

Release of carboxyfluorescein by HDL, or HDL apolipoprotein (see Figs. 3 and 4) is most easily explained in terms of an irreversible interaction in which lipid and protein are used up in a recombination process and become unavailable for further interaction. The sequence of experiments shown in Figs. 5–7 demonstrates this point. In the experiment of Fig. 5, the vesicles were passed through T_m with a concentration of HDL apolipoprotein sufficient to release about 35% of the carboxyfluorescein. The sus-

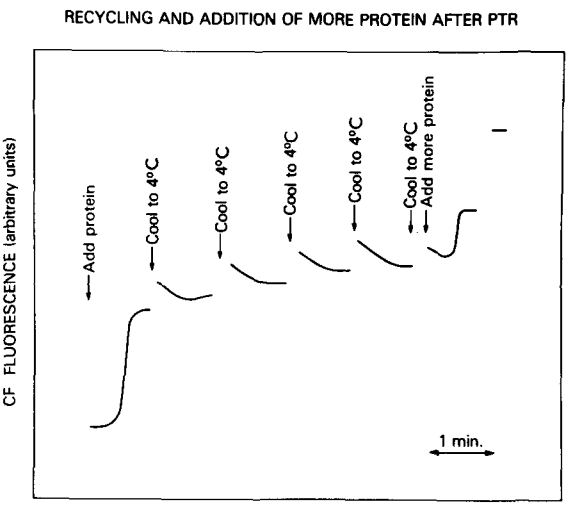

Fig. 5. Repeated temperature scans with small unilamellar DPPC vesicles and 0.029 μM HDL apolipoprotein. After the first scan, there was little additional release of carboxyfluorescein in subsequent scans until more HDL apolipoprotein was added to bring the final concentration to 0.058 μM. The downward slants in scans 2–5 reflect the temperature dependence of carboxyfluorescein (CF) fluorescence. The vesicle lipid concentration was 36 μM. Temperature profiles are not shown.

pension was then repeatedly cooled to ice temperature and again passed through T_m. The additional scans produced little release. When the original amount of protein was again added, about 37% of the remaining encapsulated carboxyfluorescein was released in the next scan. The experiment shown in Fig. 6a ruled out the trivial possibility that the results shown in Fig. 5 had been due to denaturation of HDL apolipoprotein during its brief exposure to temperatures over 40°C, or to its loss on the walls of the cuvette: when the HDL apolipoprotein was cycled twice through T_m alone and then added to the vesicles, there was little or no change in its capacity to promote release of carboxyfluorescein. Similarly, the possibility of an intrinsic change in the vesicles after first passage through transition was ruled out by

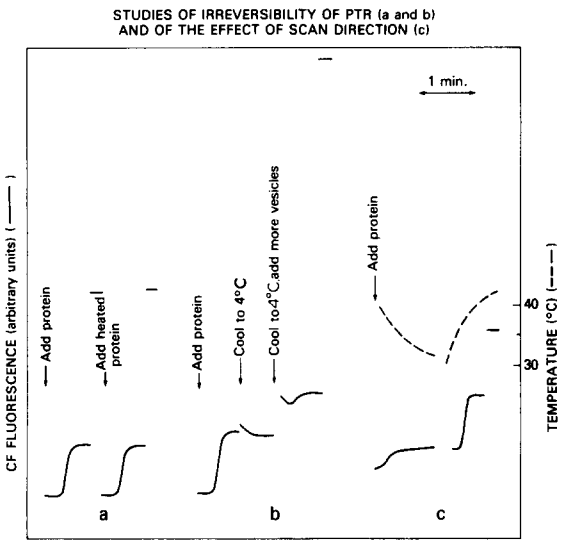

Fig. 6. Studies related to the mechanism of phase transition release (PTR) with small unilamellar DPPC vesicles and HDL apolipoprotein. (a) Two cycles of the protein through T_m before its addition to the vesicles made no difference in the amount of release. (b) HDL apolipoprotein was added to produce partial release, and a second scan produced little additional release. More vesicles were then added in small volume to double the lipid concentration. The resulting small additional release was just that expected for spontaneous release without protein. Hence, there was no protein available to enhance the release from fresh vesicles. (c) The vesicles were heated to 50°C and the protein then added. A downward scan through T_m produced little release; an upward scan then produced the expected release. The lipid and protein concentrations were 40 and 0.027 μM, respectively. CF, carboxyfluorescein.

cycling the vesicles twice and then finding the expected phase transition release on addition of HDL apolipoprotein (not shown; see Fig. 5 for analogous experiment). The experiment in Fig. 6b indicates that HDL apolipoprotein, once combined with vesicles in phase transition release, causes little or no release from additional vesicles freshly added to the suspension.

Scans at different speeds (Fig. 7) yielded essentially identical phase transition release, though the shape of the curve in (a) indicates a few percent additional release during the second scan. To use phase transition release for kinetic studies of such a fast interaction it will be necessary to use stopped-flow or temperature-jump techniques. Other experiments (unpublished data) indicate that prior binding to the gel phase is required for phase transition release. At the lipid and protein concentrations used here, binding is complete within a few seconds; at lower concentrations it is sometimes necessary to incubate the components together below T_m to obtain maximum phase transition release.

A further indication of the irreversibility of interaction was provided by experiments in which an excess of dimyristoylphosphatidylcholine small unilamellar vesicles not containing carboxyfluorescein was added to the suspension. The dimyristoylphosphatidylcholine, with a transition temperature of 23°C, interacted with the HDL at low temperatures and made it unavailable for release of carboxyfluo-

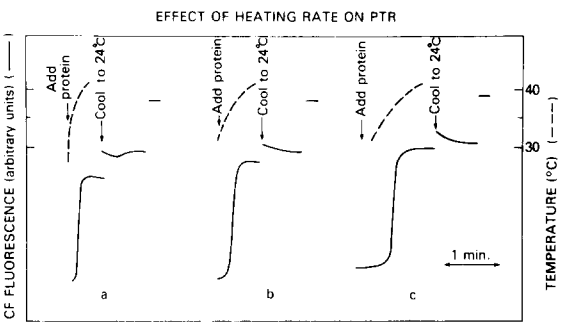

Fig. 7. Effect of scan rate on phase transition release (PTR) with small unilamellar DPPC vesicles. At 37°C, the rates were approx. 36°C/min (a), 18°C/min (b) and 14°C/min (c). Only in (a) did a second scan show additional release. Our routine practice is to do phase transition release scans at rates approximately as in (c). The DPPC and HDL apolipoprotein concentrations were 40 and 0.027 μM, respectively. CF, carboxyfluorescein.

rescein from DPPC vesicles in the suspension (data not shown). Addition of dioleoylphosphatidylcholine vesicles (with no transition temperature in the relevant range) also blocked HDL-induced release from DPPC vesicles, even though there was little release from the dioleoylphosphatidylcholine vesicles themselves (data not shown). This finding gave us our first evidence of a second type of lipid-apolipoprotein interaction, a binding which does not require the phase transition and does not cause solute release (unpublished data).

Dependence of phase transition release on direction of temperature scan

Surprisingly, upward scans through T_m produced much more release than did downward scans (Fig. 6c).

Does phase transition release involve a significant disruption of the vesicle bilayer?

We encapsulated [^{3}H]inulin (M_r. approx. 5 500) in small unilamellar DPPC vesicles along with the carboxyfluorescein (M_r 373). After passage through T_m in the presence of sufficient concentrations of HDL apolipoprotein to cause partial release, the suspensions were chromatographed on Sephadex G-200. As shown in Fig. 8 and Table I, release of the two solutes was commensurate. Therefore, the release process must involve a membrane perturbation sufficient to permit equal escape of compounds differing greatly in size and in diffusion coefficient. This finding strongly suggests an all-or-none release, especially in light of large differences we find in rates of spontaneous carboxyfluorescein and inulin release from fluid phase vesicles (data not shown). To test this supposition, we did the experiments described next.

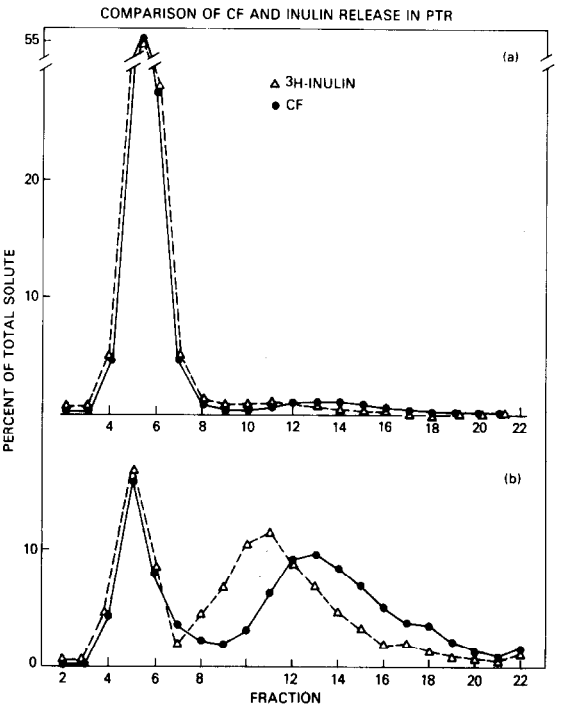

Fig. 8. Comparison of carboxyfluorescein and [^{3}H]inulin release in phase transition release. (a) Vesicles passed through transjtion without HDL apolipoprotein; (b) vesicles passed though transition with 0.3 μM HDL_3 apolipoprotein. Each suspension was then eluted with NaKCl-H on a Sephadex G-200 column. Fractions 4–6 represent the void volume peak, in which vesicles appear. The values plotted for carboxyfluorescein are those measured after addition of Triton. The free inulin elutes somewhat earlier than free carboxyfluorescein, presumably because the inulin is larger. The lipid concentration was 1.4 mM. A more extensive set of results is summarized in Table I. PTR, phase transition release; CF, carboxyfluorescein.

Is the release process an all-or-none event?

Small unilamellar vesicles were formed in 92 mM carboxyfluorescein. Portions (100 μl) of the suspension were passed through T_m, with or without addition of enough HDL apolipoprotein to produce partial release. Each sample was then cooled to room temperature and 90 μl eluted with NaKCl-H on a Sephadex G-25 column. As the vesicle peak eluted from the column, approx. 100 μl was dripped directly into a small cuvette, and the fluorescence determined within a few seconds (pre-Triton value). Triton was then added and a second reading taken. The percentages of quenching thus obtained after partial release and chromatographic separation of released dye could be compared with those found immediately after formation of vesicles in different concentrations of carboxyfluorescein (Fig. 9). If carboxyfluorescein release were a partial leakage from each vesicle, the quenching should have decreased as the fractional release increased (see Appendix for calculations). The experimental points should then have followed the solid curve in Fig. 9. However, Fig. 9 indicates that the quenching changed little with partial release, consistent with an all-or-none process in which vesicles not perturbed still contained their full

TABLE I

COMPARISON OF CARBOXYFLUORESCEIN AND [^{3}H]INULIN RELEASE IN PHASE TRANSITION RELEASE

The results indicate commensurate release of the two markers. See text for protocol.

	Final percent of solute outside of vesicles [a]	
	Carboxyfluorescein	[^{3}H]Inulin
No phase transition release, 0.30 μM HDL_3 apolipoprotein	6	2
Phase transition release, no HDL_3 apolipoprotein	6	4
Phase transition release, 0.15 μM HDL_3 apolipoprotein	57	54
Phase transition release, 0.30 μM HDL_3 apolipoprotein	65	68

[a] DPPC concentration was 1.4 mM in these experiments. PTR, phase transition release.

complement of dye after phase transition release.

We should note in passing an additional, slightly more complex possibility which might have been observed but was not. If phase transition release had involved partial leakage from each vesicle and also vesicle-vesicle fusion producing spherical but larger recombinants, the points would have been expected to fall above the line because the increased ratio of internal volume to lipid would have necessitated dilution of the dye.

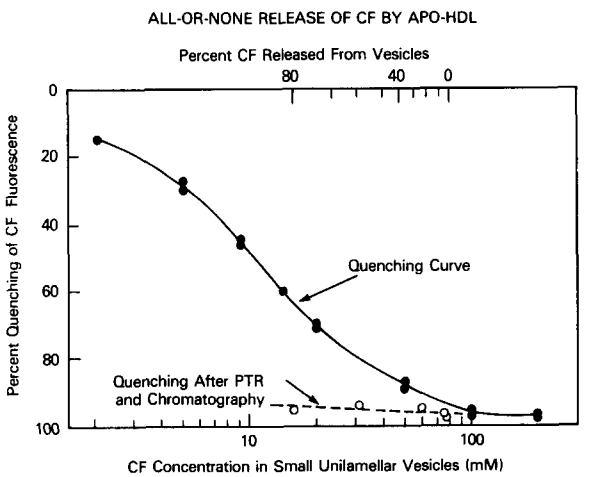

Fig. 9. All-or-none release of carboxyfluorescein by HDL apolipoprotein. The solid curve and filled circles represent the quenching of carboxyfluorescein in small unilamellar vesicles at each indicated concentration. The open circles represent quenching ratios after phase transition release, as explained in Results. PTR, phase transition release; CF, carboxyfluorescein; Apo, apolipoprotein.

Promotion of phase transition release by serum components other than HDL

It is clear that liposomes are broken up in vivo and their contents released by serum components, principally the lipoproteins [43–45]. We therefore asked whether the other lipoprotein fractions would promote phase transition release as do HDL and HDL apolipoprotein. The results in Table II indicate that all

TABLE II

RELATIVE EFFICACY OF HUMAN PLASMA FRACTIONS IN PHASE TRANSITION RELEASE

Plasma fraction	Protein required for 50% carboxyfluorescein release [a] (μg/ml)	Protein concentration in plasma [b] (μg/ml)
VLDL	10	170
IDL	9	80
LDL	15	520
HDL	8	1100
d = 1.21 infranate	210	≈30000
Albumin [c]	>4000	40000
Whole plasma	0.4% [d]	

[a] Vesicle lipid concentration 48 μg/ml. See Appendix for calculation.

[b] As measured by the method of Lowry et al. on serum fractions used in this experiment.

[c] Crystalline bovine serum albumin (Miles Laboratories). Crude fraction V albumin preparations often contain significant phase transition release-inducing contaminants [45].

[d] That is, a 250-fold dilution of plasma will release 50% of carboxyfluorescein from this concentration of vesicles.

of the lipoprotein fractions promote phase transition release to roughly the same extent per unit of protein weight. In the case of LDL, whose major protein component (apolipoprotein B) is bound very tightly and does not exchange among lipoprotein particles, we wondered whether the effect might be due to contamination with other apolipoproteins. This possibility was ruled out by experiments with a narrower centrifugation fraction of LDL (d = 1.02–1.05), centrifuged a second time to remove residual contaminants. This preparation showed no discernible proteins other than apolipoprotein B on SDS-polyacrylamide gels, but it promoted phase transition release in quantitatively and qualitatively the same way as did the initial LDL fraction. The nature of the interaction between LDL and vesicles remains to be elucidated.

Effect of liposome type on phase transition release

Fig. 11 shows phase transition release profiles for peak I liposomes from a Sepharose 4B column (Fig. 10). These liposomes are larger than the small unilamellar vesicles, and largely multilamellar, but their structure has not otherwise been well characterized. In the absence of lipoprotein, there was a major spontaneous release. Addition of lipoprotein somewhat increased the release but only to a peculiar sharp cut-off well short of 100%. Clearly, some of the carboxyfluorescein releasable by Triton was protected from the effects of lipoprotein, perhaps on the interior of lipid structures of a different nature than the standard vortex-mixed multilamellar vesicles or small unilamellar vesicles. The secondary increase in phase transition release seen with high concentrations of HDL may have been due to contamination of the fraction I liposomes with small unilamellar vesicles. Solid-phase small unilamellar vesicle preparations change with time at both 4°C and room temperature. The first indications of this are high spontaneous release in phase transition release and inability to obtain 100% release with high concentrations of lipoprotein.

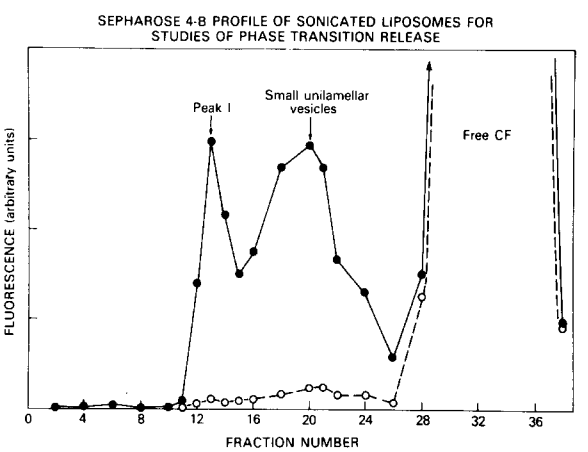

Fig. 10. Sepharose 4B profile of sonicated DPPC vesicles. (○) pre-Triton fluorescence, indicating carboxyfluorescein outside vesicles plus the small residual fluorescence of 100 mM carboxyfluorescein inside vesicles; (●) post-Triton fluorescence, indicating total carboxyfluorescein. The lipid (25 mg) was sonicated at setting 2 for only 20 min, then 'annealed' at 45°C for 30 min. Fractions were 1 ml. CF, carboxyfluorescein.

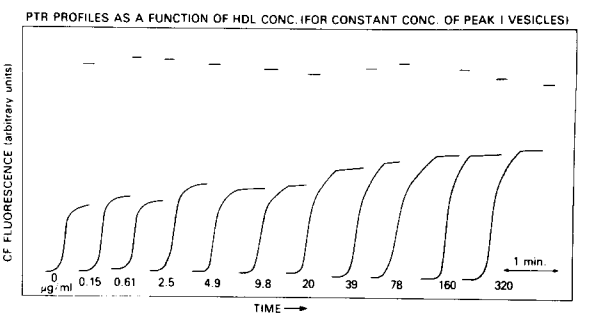

Fig. 11. Phase transition release profiles for Sepharose peak I liposomes, as a function of HDL concentration. See legend to Fig. 2 for details. The profiles show two components at high protein concentrations, and a sharp cut-off well short of 100% release. The carboxyfluorescein concentration of the suspension was 1.6 μM. CF, carboxyfluorescein.

When multilamellar vesicles were made with 100 mM carboxyfluorescein, there was almost complete spontaneous release in NaKCl-H, starting at a temperature well below T_m. As indicated in Fig. 12, much of this release could be prevented by osmotic protection. Multilamellar vesicles prepared in a similar way with 70 mM carboxyfluorescein showed little spontaneous phase transition release in NaKCl-H, though they did show major release in hypoosmolar solution. Fig. 13b shows phase transition release scans for a preparation stable in NaKCl-H. There was an initial small spontaneous release, little release on recycling, and then only a slow, small effect of HDL apolipoprotein. These observations suggest that the protein did not have access, at least on this time scale, to inner

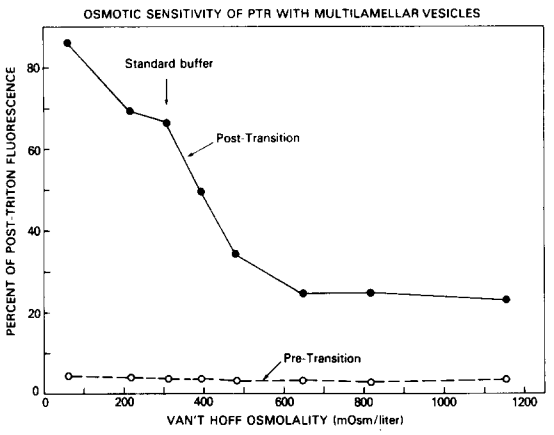

Fig. 12. Osmotic sensitivity in phase transition release (PTR) of multilamellar vesicles prepared with 100 mM carboxyfluorescein. Below T_m, the vesicles were stable at all osmolarities tested, as indicated by the low pretransition fluorescence. On passage through T_m without protein, there was a large release in hypotonic media. The major part of this release was prevented by osmotic protection. The total carboxyfluorescein concentration in the suspension was 1.6 μM.

lamellae of the liposomes. Small unilamellar vesicles, on the other hand, released completely with sufficient protein and were able, even near T_m, to withstand considerable osmotic pressure differences generated across their bilayers by high internal carboxyfluorescein concentrations. This resistance is probably due to their low radius of curvature.

Large unilamellar vesicles formed by reverse phase evaporation were osmotically sensitive at T_m but much less so at lower temperatures, hence a considerable osmotic release occurred at T_m if the osmotic pressure in the vesicle exceeded that in the medium (data not shown). Osmotically balanced vesicles (Fig. 13a) showed a small amount of spontaneous release in an initial scan without lipoprotein, no further release in a second scan, and then large fractional release after addition of HDL apolipoprotein.

Discussion

To illustrate the characteristics of phase transition release as a process and as a method, we have here

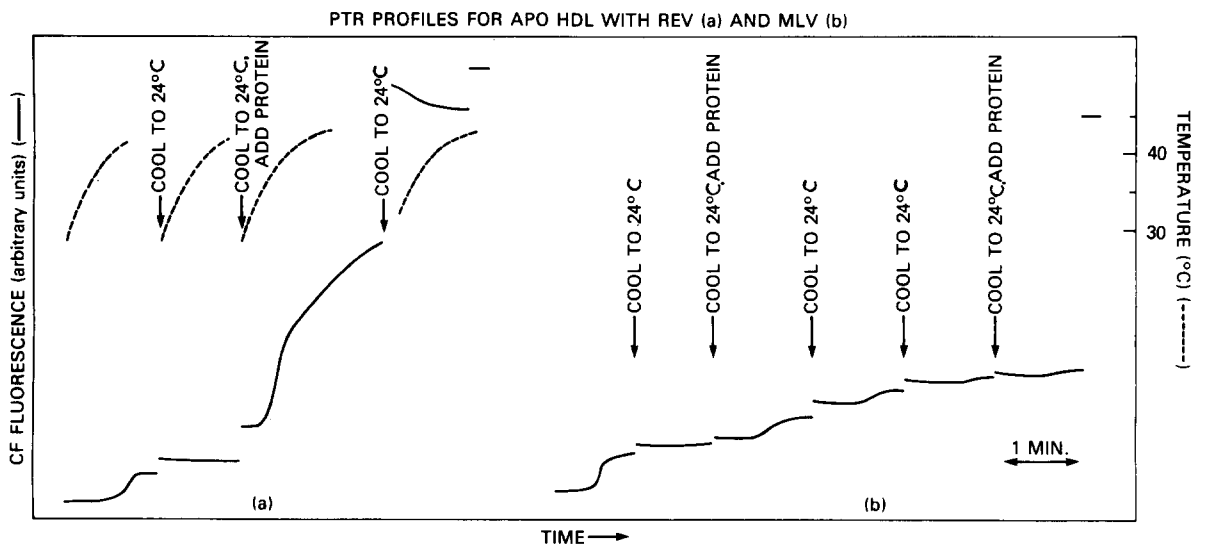

Fig. 13. Phase transition release (PTR) characteristics of reverse phase evaporation vesicles (a) and vortex-mixed multilamellar vesicles (b). The reverse phase vesicles (REV) showed a small spontaneous release, with no further release on recycling through T_m. After addition of HDL apolipoprotein (APO HDL) to a final concentration of 52 μg/ml (lipid/protein ratio of 23 : 1), there was a large release. Recycling indicated considerable additional release even before the next upward temperature scan. The multilamellar vesicles (MLV) showed a small spontaneous release on the first scan and only a slight additional release on addition of HDL apolipoprotein to a final concentration of 52 μg/ml. This release clearly continued for at least two scans, and addition of more protein yielded no further effect. On this time scale, the interaction was slow and incomplete. CF, carboxyfluorescein.

considered the interactions of HDL and HDL apolipoprotein with phospholipid vesicles. We focused on small unilamellar vesicles because they show the clearest pattern of interaction with lipoproteins. Below the phase transition temperature, there is very little release of carboxyfluorescein, even in the presence of HDL or HDL apolipoprotein; above the transition temperature there is a slow but significant release, enhanced by the presence of HDL or HDL apolipoprotein; at the transition, the rate of release is dramatically increased with addition of the protein. This fast release forms the basis of phase transition release as a method for studying lipid-protein interactions. Detailed observations with phase transition release permit a number of inferences about the phenomenology and mechanisms of interaction. In the cases of HDL and HDL apolipoprotein:

(1) The release of contents goes essentially to completion within a few seconds, and the extent of release is affected very little by rate of heating (within the range of rates experimentally accessible with the apparatus used). Additional cycles of temperature through T_m produce little additional release. These observations suggest a definable limit to the combining power of the protein.

(2) The interaction is irreversible, as indicated by the lack of additional release with repeat scans, even if fresh vesicles are added. As would be expected, addition of fresh protein after partial release leads to additional phase transition release.

(3) HDL apolipoprotein is about 20-times as powerful as native HDL in promoting phase transition release. HDL apolipoprotein induces 50% release at a vesicle lipid/protein molar ratio of about 3 400 : 1. Equivalent release is obtained with HDL only at lipid/protein ratios of about 160 : 1.

Using KBr density gradients (data not shown), we find that interaction of HDL with the vesicles involves a homogeneous recombination of the HDL particle (with an amount of lipid that approximately doubles its lipid content). Therefore, the limited ability of native HDL to interact with the vesicles in phase transition release cannot represent the effect of a small proportion of the protein molecules behaving like free HDL apolipoprotein. Furthermore, we find that the recombinant structures formed with HDL apolipoprotein contain lipid/protein ratios approximating those that would be expected from the phase transition release titration curve in Fig. 4. Thus, the vastly different lipid-combining powers of native and HDL apolipoprotein appear to reflect differences in the types of recombinant structures they can form with DPPC vesicles.

Disc-shaped recombinants at molar lipid/protein ratios of about 100 : 1 have been studied frequently in the past few years [14–29], generally with dimyristoylphosphatidylcholine liposomes. However, phase transition release is virtually complete at ratios of more than 1 000 : 1, so a recombinant different from the usual disc appears to be forming. We find, in fact, that phase transition release with apolipoprotein A1 involves formation of vesicular recombinant particles larger than the original vesicles (unpublished results). Jonas and coworkers [28,29] have described what may be similar recombinants with dimyristoylphosphatidylcholine vesicles.

(4) Phase transition release induced by HDL apolipoprotein is an all-or-none event. As indicated in Fig. 9, which is based on relief of the self-quenching of carboxyfluorescein, the increase in fluorescence during the scan results from total release of dye from some of the vesicles and no detectable release from the rest. We have not done the corresponding experiments with whole HDL.

(5) With HDL apolipoprotein, the disruption at the phase transition is sufficient that both carboxyfluorescein (M_r 373) and inulin (M_r approx. 5 500) are released to the same extent. This finding suggests a considerable disruption of the vesicle structure at T_m.

(6) The amount of release depends strongly on the direction of scanning through transition. If protein is added above the transition and the sample cooled, there is very little release. If the sample is then heated through transition, the usual phase transition release is seen. This asymmetry could be due to a role of the lipid 'pretransition' or to a hysteresis in the lipid melting-freeze cycle at T_m. However, studies with apolipoprotein A1 (unpublished results) suggest that the asymmetry arises from a requirement for prior binding to the gel state vesicles (a slow step) before release can take place at T_m.

Phase transition release studies are most easily done at small unilamellar vesicle lipid concentrations of about 20–80 μM. At these and higher concentrations, the percentage release depends only on the

lipid-protein stoichiometry, not on the absolute concentrations (data not shown). At lower concentrations, it is necessary to wait for the slow binding to occur after mixing of lipid and protein if stoichiometric release is to be obtained.

(7) Small unilamellar vesicles and multilamellar vesicles show very different patterns of phase transition release. Small unilamellar vesicles are convenient for phase transition release studies in that they are of relatively well defined size, show little spontaneous release at the phase transition, and release contents rapidly and completely at T_m in the presence of sufficient protein. In addition, they do not release contents under osmotic stress, a feature of particular importance for assessing the effect on protein-lipid interaction of changes in ionic strength with denaturants such as urea or guanidine hydrochloride. Multilamellar vesicles, on the other hand, are osmotically sensitive. They appear able to withstand considerable osmotic stress in the gel state but to release contents spontaneously at T_m in response to osmotic imbalance. Different batches of multilamellar vesicles give quite different results in phase transition release, depending on details of the composition of the internal and external milieux and, presumably, on the size of the particles present. Both multilamellar vesicles and Sepharose peak I vesicles release only part of their carboxyfluorescein in phase transition release no matter how much HDL or HDL apolipoprotein is added. This limitation probably results from a relative protection of the inner lamellae of the structures. Below T_m, small unilamellar vesicles of DPPC transform spontaneously over a period of days into larger unilamellar vesicles of about 600–800 Å diameter [46]. Elsewhere we will present data suggesting that at T_m the apolipoproteins trigger a greatly accelerated version of this transformation, perhaps correlated with the release process. However, the observation of phase transition release with multilamellar vesicles and reverse-phase evaporation vesicles indicates that phase transition release does not simply reflect an instability of the strained small unilamellar vesicle structure.

(8) All of the lipoprotein fractions and also the nonlipoprotein fraction of plasma promote phase transition release. Since the percentage phase transition release is approximately constant for a given lipid/protein ratio, the data in Table II can be used to estimate the amount of release expected at T_m for any specified load of liposomes injected in vivo. This is an important consideration in the pharmacology of 'temperature-sensitive' liposomes [47,48].

(9) Most proteins do not induce phase transition release. Whereas HDL apolipoprotein at 0.41 μg/ml causes 50% release from vesicles containing 60 μM lipid, the following proteins at concentrations greater than 100 μg/ml induce no significant phase transition release with the same vesicles: bovine serum albumin (crystalline), ovalbumin, rabbit immunoglobulin G (and its $F(ab)'_2$ and F_c fragments), rabbit immunoglobulin M, trypsin, chymotrypsin, pronase, hemoglobin, and hen lysozyme. Among the cytoskeletal elements, synexin, ankyrin, myosin, and the microtubule-associated proteins similarly have no effect. Tubulin, on the other hand, does induce phase transition release (50% release at molar lipid/tubulin ratios of 375 : 1), but the phenomenology is very different from that seen with the lipoproteins [30]. For one thing, the release is not an all-or-none process, and for another, there is no apparent asymmetry with direction of scan. Actin also induces phase transition release.

At this point we can only speculate about the molecular details of the lipid-protein interaction underlying phase transition release. With both the lipoproteins and tubulin, phase transition release appears to reflect a stable insertion of protein into the bilayer, probably a result of increased lateral compressibility of the lipid structure at the phase transition. Solute release might indicate penetration of the protein sufficient to perturb both leaflets of the bilayer. It is clear, however, that strong interaction of protein with the bilayer can take place without release. We find that a hepatic receptor protein for asialoglycoproteins interacts hydrophobically with the lipid either below, above, or near T_m but does not induce phase transition release [31].

Phase transition release provides an easily accessible new parameter of protein-bilayer interaction and also a means for reconstituting relatively water-soluble proteins into vesicles without detergent. Only further experience with a variety of proteins will yield an appropriate perspective on its utility in these two contexts, and on its possible relation to mechanisms of physiological protein insertion into bilayers.

Appendix

For most purposes the extent of phase transition release can be estimated reasonably well by eye. Here, however, we present explicit calculations from the data. Each fluorescence reading (F) is first corrected to 45°C by an empirical expression obtained from fluorescence measurements on free carboxyfluorescein: $F' = F(1.437 - 0.0097T)$, where T is the temperature (°C). The corrected post-Triton fluorescence is multiplied by an additional factor of 1.04 (because of the dilution with Triton) to obtain F'_t. Then, a quite general expression for the carboxyfluorescein fluorescence (F') observed at any moment during a scan is

$$F' = [x + \alpha(1 - x)] \cdot F'_t \quad (1)$$

where x is the fraction of carboxyfluorescein outside of the vesicles and α is the quenching factor for carboxyfluorescein in the vesicles (see Fig. 9). Rearranging Eqn. 1, we have

$$x = \left(\frac{F'}{F'_t} - \alpha\right)/(1 - \alpha) \quad (2)$$

Before Eqn. 2 can be used, it is necessary to decide on a model for the release process. If the mechanism were all-or-none, α would remain constant (at about 0.05 for vesicles containing 10 mM carboxyfluorescein), since dye remaining in the unaffected vesicles would still be at 100 mM. If the process were a permeation involving all vesicles equally, α would be a function of the fraction of dye released (see Fig. 9), and an iterative calculation would be required. Based on experiments noted in the text, it appears that spontaneous release away from the transition temperature is a permeation, but that the release induced by apolipoprotein at T_m is all-or-none. Hence, we will consider that α is constant (and approx. 0.05 for 100 mM carboxyfluorescein in small unilamellar vesicles). In Figs. 3 and 4, the units on the ordinate are 'percent of post-Triton fluorescence' after volume and temperature correction (i.e., F'/F'_t). The values are thus a good approximation to x if α is small, but the calculations do not presume prior knowledge of the mechanism of release.

The most appropriate parameter to use for fractional release induced (see Fig. 2 for definitions) by protein is: $(F'_b - F'_a)/(F'_t - F'_a)$. The assumptions underlying use of this parameter are (a) that the spontaneous release at the transition is a permeation, or (b) if it is all-or-none, that the lipid of emptied vesicles remains available to bind apolipoprotein.

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