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Effect of Phosphatidylethanolamine on the Properties of Phospholipid-Apolipoprotein Complexes†

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ABSTRACT: Plasma high density lipoproteins (HDL) are synthesized in intestinal mucosal cells and hepatocytes and are secreted into the blood. Factors influencing the structure and function of these HDL, such as lipid and protein composition, are poorly understood. It appears, however, that intracellular, discoidal HDL are enriched, relative to plasma HDL, in phosphatidylethanolamine (PE), a phospholipid known to generate unusual, nonbilayer structures of putative physiological significance. Although incubation of dimyristoylphosphatidylcholine (DMPC) with apolipoprotein A-I at the gel–liquid crystalline phase transition temperature results in the spontaneous formation of lipid–protein complexes, the presence of proportionately small amounts of PE prevents the formation of such complexes, suggesting that PE profoundly alters the phase properties of the phospholipid bilayers. However, by using a detergent-mediated method for the formation of PE-rich model nascent HDL from phospholipids and apolipoprotein A-I, lipid–protein complexes containing as much as 75% DLPE could be formed, thus demonstrating that the presence of PE causes a kinetic, rather than a thermodynamic, barrier to spontaneous complex formation. The products contained a DLPE:DMPC molar ratio similar to that of the initial incubation mixture; however, as the mole percentage of DLPE increased, the products became less heterogeneous, the buoyant density of the products increased, and the Stokes diameter of the products decreased. Similar results were obtained when dimyristoylphosphatidylethanolamine (DMPE) and dipalmitoylphosphatidylethanolamine (DPPE) were employed in lieu of DLPE. Electron microscopy of complexes containing DLPE and DMPC at a 1:1 molar ratio showed that these particles possessed a discoidal, bilayer morphology similar to that seen with complexes containing only phosphatidylcholine. PE increased the susceptibility of the particles to denaturation by guanidine hydrochloride and caused a much sharper endotherm at the phase transition as shown by differential scanning calorimetry; however, circular dichroism studies showed that the A-I secondary structure was similar in complexes with DMPC alone and when complexed with a mixture of DMPC and DLPE. The results from these experiments suggest that the presence of DLPE in a mixture with DMPC diminishes the interaction between protein and lipid in these complexes. We conclude that under appropriate conditions, phosphatidylethanolamines can be incorporated into model nascent HDL, but the presence of PE significantly alters certain of the physical properties of these HDL, such as size and density, compared to those prepared with phosphatidylcholines alone.

Plasma lipoproteins are soluble, pseudomicellar, lipid–protein assemblies that transport lipids to and from tissues and mediate several reactions of lipid metabolism. Plasma high density lipoproteins (HDL)[1] are spherical particles with a diameter range of 8.5–12.0 nm (Anderson et al., 1977). Human plasma HDL are composed of a neutral lipid apolar core consisting of cholesteryl ester and triglyceride that is solubilized by an outer surface of amphoteric lipids and apolipoproteins A-I, A-II, and C. Transitory precursors of plasma HDL, termed nascent HDL, appear to be secreted in three discrete forms: as phospholipid-rich, discoidal structures, as phospholipid-rich, small, spherical structures, and as free apolipoproteins not bound to lipid (Marsh, 1976; Hamilton et al., 1976; Bisgaier

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[1] Abbreviations: HDL, high density lipoproteins; apo A-I, apolipoprotein A-I; PC, phosphatidylcholine; PE, phosphatidylethanolamine; DPPC, dipalmitoylphosphatidylcholine; DMPC, dimyristoylphosphatidylcholine; DLPE, dilauroylphosphatidylethanolamine; DMPE, dimyristoylphosphatidylethanolamine; DPPE, dipalmitoylphosphatidylethanolamine; A-I, apolipoprotein A-I; GGE, pore limit gradient gel electrophoresis; DMS, dimethyl suberimidate; SDS-PAGE, polyacrylamide gel electrophoresis in sodium dodecyl sulfate; LCAT, lecithin: cholesterol acyltransferase.

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& Glickman, 1983; Schonfeld & Pfleger, 1974).

Little is known about the process by which apolipoproteins and lipids assemble to form the various types of nascent HDL or about the lipid–protein interactions which dictate the dynamic structure and function of nascent HDL. It is known, however, that the percentage of phosphatidylethanolamine (PE) as a fraction of total phospholipids in intracellular HDL isolated from chick Golgi contents was higher than in serum HDL (Banerjee & Redman, 1983). In particular, the weight ratio of PE to phosphatidylcholine (PC) in chick hepatic Golgi HDL is 0.45:1 whereas in chick serum HDL the ratio was found to be 0.28:1. In contrast, the PE:PC weight ratio in rat serum HDL (Swaney et al., 1987) and human serum HDL (Scanu, 1972) is much lower, at 0.02:1 and 0.04:1, respectively. Similarly, the percentage of PE in intracellular VLDL is higher than the percent of PE found in plasma VLDL (Howell & Palade, 1982).

The relative enrichment of PE in intracellular lipoproteins is interesting in light of the tendency of this lipid to assemble into nonbilayer structures (hexagonal phase) as opposed to the bilayer structures formed by phosphatidylcholines. Furthermore, it has been recently reported that normal human plasma contains a population of very low density lipoproteins (VLDL) comprising 25% of the total VLDL apo B, which are likewise enriched in PE, at a level 2.6 times the content in the remaining VLDL (Fielding & Fielding, 1986). It is of interest that Haase and Stoffel found that when the A-I gene was expressed in oocytes, the protein was associated mainly with newly synthesized phosphatidylethanolamine (Haase & Stoffel, 1988). To date, however, there have been no studies of the impact of elevations in PE content on the physical properties of lipoprotein particles.

A few studies have been performed that evaluate the substrate properties of PE with enzymes that metabolize lipoprotein phospholipid. For example, numerous studies have shown that both in vivo (Landin et al., 1984; Landin & Nilsson, 1984) and in vitro (Laboda et al., 1986; Ehnholm et al., 1975; Sundarem et al., 1978) PE is a significantly better substrate for the phospholipolysis activity of hepatic lipase than PC. Similarly, Pownall et al. (1985) found that PE was the best phospholipid substrate for LCAT when incorporated into a reassembled HDL composed mostly of diether analogues of PC. The preferential hydrolysis of PE, with loss of the soluble lysoPE from the lipoprotein surface, could explain the reduced content of PE found in serum lipoproteins.

In short, several studies suggest that PE may play an important role as a substrate for key enzymes in the metabolism of lipoproteins, especially HDL, and that certain subpopulations of the lipoproteins, perhaps newly secreted forms, are relatively enriched in this phospholipid species. However, there have been no systematic investigations into the effects of PE on the properties of lipid–protein complexes, in part because of difficulties in forming these complexes. This study was undertaken to determine the limitations upon the incorporation of PE into model HDL and to ascertain the effects of PE on the particles that were obtained.

Materials and Methods

Materials. Plasma was obtained from male and female human subjects. HDL was isolated from plasma by sequential flotation ultracentrifugation (Schumaker & Puppione, 1986). Apo A-I was isolated from HDL apolipoproteins by Sephadex G-150 (Pharmacia) column chromatography (Nunez & Swaney, 1984).

5,5′-Dithiobis(2-nitrobenzoic acid) was purchased from Pierce Chemical Co. (Rockford, IL). Bio-Beads SM-2 were purchased from Bio-Rad Laboratories (Richmond, CA). Sodium phosphotungstate and Formvar carbon-coated copper grids were purchased from Polysciences, Inc. (Warrington, PA). Phospholipids were purchased from Avanti Polar Lipids (Birmingham, AL). Sodium cholate was purchased from Sigma (St. Louis, MO).

Thin-layer chromatography using Whatman Linear-K 5D Preadsorbant TLC plates was employed to verify the purity of the phospholipids and sodium cholate. Phospholipids were separated by using a mobile-phase mixture of chloroform/methanol/water (65:25:4 v/v/v). For resolution of cholate a mobile-phase mixture of trimethylpentane/ethyl acetate/acetic acid (5:25:0.2 v/v/v) was used. After separation was achieved, the solvent was evaporated and the plate was exposed to iodine vapors. All reagents checked for purity yielded a single spot and were used without further purification.

Preparation of Lipid–Apo A-I Complexes. PE-containing lipid–protein complexes were routinely prepared by using a modification of a method for preparing phosphatidylcholine-containing complexes that we recently described (Bonomo & Swaney, 1988). In brief, this method involves codispersion of the lipid and protein components with cholate, which was then removed by a batch chromatographic process employing an adsorption column containing Bio-Beads SM-2 and elution by centrifugation. The method was modified by increasing the time of centrifugation for the removal of the beads from the incubation mixture from 2 min to 30 min whenever the mixture contained PE to pellet unreacted lipid, thus clarifying the eluate. In preparations of lipid–protein complexes by this method, as well as by cholate dialysis or spontaneous reaction at the phase-transition temperature, some batch-to-batch variation was noticed in the relative staining of higher molecular weights bands in gradient gel electrophoresis.

Characterization of Phospholipid–Apo A-I Complexes. To quantitate protein in the presence of lipid, a modification of the Lowry procedure (Lowry et al., 1951) was used (Markwell et al., 1978). Total phospholipid phosphorus was determined by acid digestion and colorimetry (Marinetti et al., 1959). To determine the phosphatidylcholine concentration, an enzymatic assay kit (Wako Pure Chemical Industries, Ltd., Osaka, Japan; phospholipids B kit) was used. In this procedure, phosphatidylcholine was hydrolyzed by phospholipase D to yield phosphatidic acid and choline. The liberated choline was subsequently oxidized by choline oxidase to yield betaine with the simultaneous production of hydrogen peroxide. The hydrogen peroxide was oxidatively coupled to 4-aminoantipyrine and phenol to yield a chromogenic quinone with an adsorption maxima at 505 nm. The concentration of phosphatidylethanolamine was derived by subtracting the phosphatidylcholine concentration from the concentration of total phospholipid phosphorus.

The size of lipid–protein complexes was determined by pore limit gradient gel electrophoresis (GGE). Electrophoresis was performed by using precast PAA 4/30 gels purchased from Pharmacia (Piscataway, NJ) with a Pharmacia gel electrophoresis Apparatus-2/4 cooled to 7.0 °C and electrophoresis was conducted for a total of 3000 V·h. Protein standards (Pharmacia) for estimating molecular weight or Stokes diameter were purchased from Pharmacia and the following values for Stokes diameters and estimated molecular weights were used: thyroglobulin, 17.0 nm, 669 kD; ferritin, 12.2 nm, 440 kD; catalase, 10.2 nm, 232 kD; lactate dehydrogenase, 8.1 nm, 140 kD; and albumin, 7.1 nm, 67 kD (Blanche et al., 1981).

To determine the extent of lipid–protein complex formation and the protein stoichiometry of the complexes, a cross-linking reagent, dimethyl suberimidate (20 mg/mL in 1 M triethanolamine–HCl, pH 9.7), was added to aliquots of the samples and allowed to react at room temperature for 2 h (Swaney & O'Brien, 1978). The cross-linked self-associated forms of lipid-free apo A-I range from monomers to pentamers and these bands were used as markers to determine the number of protein chains per particle in the lipid–protein complexes.

The buoyant density of lipid–protein complexes was determined by density gradient ultracentrifugation (Kelley & Kruski, 1986).

Stability to denaturation was studied by fluorescence emission wavelength measurements made at 25 °C in a Perkin-Elmer LS-5 fluorescence spectrophotometer using an excitation wavelength of 278 nm. The sample dilution buffer was 0.045 M NaCl, 0.03 M $NaHCO_3$, pH 8.5 (Swaney, 1983). A solution of apo A-I (initially 0.1 mg/mL buffer) or one of the phospholipid–apo A-I complexes (initially 0.025 mg of apo A-I/mL) was placed in a cuvette and the emission spectrum between 280 and 400 nm was recorded after each addition of an 8.0 M guanidine hydrochloride solution (solid guanidine hydrochloride was used to achieve concentrations above 5 M). Since the emission curves appeared symmetrical around the midpoint of the emission maximum, the maximum wavelength was determined by bisecting a horizontal tie line drawn close to the peak maximum. Curves were drawn to best approximate a sigmoidal profile as shown previously for these types of data (Swaney, 1983).

The completeness of lipid incorporation into lipid–protein complexes in the Bio-Beads eluates was evaluated by subjecting the eluate to a density gradient separation in a KBr gradient using an SW41 rotor. The gradient was prepared by successively layering 3.0 mL of a d = 1.21 g/mL KBr solution, 2.0 mL of d = 1.125 g/mL KBr, 2.0 mL of d = 1.063 g/mL KBr, and eluate (0.4 mL) adjusted to d = 1.030 g/mL with KBr and by filling the tube with 1.4 mM NaCl, 0.01% EDTA, pH 7.0. After centrifugation at 36000 rpm for 20 h, the tubes were punctured and fractionated into 0.68-mL portions. The protein was quantitated by the Lowry method and phosphatidylcholine was quantitated enzymatically. To obtain a semiquantitative estimation of the phosphatidylethanolamine distribution, 50-μL aliquots of the individual fractions were spotted on silica gel thin-layer plates and the plates were eluted with chloroform–methanol–acetic acid–water 25:14:4.2 (v/v/v/v) to separate phospholipid species. The plates were sprayed with fluorescamine to allow visualization of the PE-containing spots; these areas were scraped off from each sample lane into individual tubes. The fluorescent product was solubilized by the addition of methanol and the fluorescence of each fraction was measured at 475 nm with excitation at 390 nm.

Electron Microscopy. To determine the morphology of the phospholipid–apo A-I complexes, we employed a negative-stain electron microscopy technique (Forte & Nordhausen, 1986). A droplet of lipid–protein complex solution (100 μg of protein/mL) was placed onto a Formvar carbon-coated copper grid. After 20 s, excess lipoprotein solution was removed with filter paper and a droplet of 1% sodium phosphotungstate, pH 7.2, was added. After 20 s, excess stain was removed with filter paper, and the grid was immediately placed into the electron microscope chamber for study.

Circular Dichroism. The secondary structure of apo A-I in lipid–protein complexes was studied by circular dichroism by using a JASCO Model J-41A spectrophotometer. The instrument was calibrated with (+)-camphorsulfonic acid. Four replicate samples for each type of complex were prepared in two separate experiments, and for each sample four spectra were averaged. The data were analyzed by using the method of Greenfield and Fasman (Greenfield & Fasman, 1969).

Differential Scanning Calorimetry. Study of the thermal transition properties of multilamellar lipid vesicles and complexes of apo A-I with phospholipid were performed by using a high-sensitivity differential scanning calorimeter (MicroCal-2, MicroCal, Inc., Northampton, MA). The sample cell volume was 1.19 mL and heating was at a rate of 90 deg/h. Complexes of A-I and DMPC or DMPC/DLPE (1:1 mol/mol) mixtures were prepared by the Bio-Beads method: column eluates containing the protein–lipid complexes were dialyzed against phosphate buffered saline, which was used in the reference cell, and duplicate samples were purified by passage over a Superose 12B FPLC column (Pharmacia, Piscataway, NJ). The final concentration of each sample was approximately 0.5 mg of protein/mL. Multilamellar dispersions of the lipids alone (3 mg/mL) were prepared by drying the lipids as a film, adding phosphate-buffered saline, and vortexing. The calorimeter was interfaced to a computer using a DA-2 data acquisition and analysis system (MicroCal, Inc.), which was used to collect data and analyze the thermal transitions.

Results

Evaluation of the Ability of DLPE To Interact Spontaneously with Apo A-I. The evaluation of the ability of DLPE to interact spontaneously with apo A-I was based, in part, on the criterion that the spontaneous formation of soluble phospholipid–apo A-I complexes, which occurs during the incubation of apo A-I with a turbid aqueous dispersion of phospholipid, is positively correlated to the clearance of the turbidity of the codispersion (Trauble et al., 1974). The selection of DLPE for study was dictated by its phase-transition properties, since previous studies have shown that only phospholipids with phase-transition temperatures below 35 °C react spontaneously with apolipoprotein A-I (Swaney & Chang, 1980; Swaney, 1980), and DLPE is one of the few species of PE that meet this criterion. Thus, a turbid aqueous dispersion of pure DLPE (20 mM DLPE) was prepared and after preincubation at 30 °C for 1 h, apo A-I was added (140 mol of DLPE/mol of apo A-I, 1.1 mg of apo A-I/mL). Following incubation of this codispersion for 2 h at 30 °C[2] no clearing of turbidity was observed. Ultimately, a white precipitate formed, indicating that little, if any, spontaneous formation of soluble DLPE–apo A-I complexes occurred.

Previous studies from this laboratory have shown that the inclusion of DMPC, a lipid that is highly reactive with apolipoproteins, can facilitate the formation of complexes between apo A-I and phosphatidylcholines, which alone fail to interact with apo A-I (Swaney, 1980). To evaluate whether this approach could facilitate the formation of PE-containing phospholipid–apolipoprotein complexes, several turbid aqueous dispersions of phospholipid (20 mM phospholipid) that contained pure DMPC, DLPE mixed with DMPC in various proportions (5–75% DLPE), or pure DLPE were prepared and incubated for 1 h at 30 °C. Apo A-I was then added to each of the dispersions to yield a final phospholipid:apo A-I molar ratio of 140:1 (1.1 mg of apo A-I/mL), and this codispersion was then incubated for 2 h at 30 °C. The turbidity of the incubation mixture that contained DMPC and apo A-I without

[2] A temperature of 30 °C was employed since this is the phase-transition temperature of DLPE (Mantsch et al., 1983).

DLPE cleared, thus indicating that DMPC–apo A-I complexes formed. In contrast, as the proportion of DLPE in the incubation mixture increased, the degree of clearance of turbidity decreased, and a white precipitate formed whenever the proportion of DLPE exceeded 20 mol % of the total phospholipid. These results suggested that, under these conditions, there was an incomplete incorporation of the components of the incubation mixture into soluble, phospholipid–apo A-I complexes.

To evaluate the size of products formed in these incubations, an aliquot of each incubation mixture was subjected to pore limit gradient gel electrophoresis as shown in Figure 1A. Lane 1 shows the electrophoretic pattern of the products that formed during the incubation of apo A-I with an aqueous dispersion of DMPC alone. Several major bands were observed that had R_f values between 0.34 and 0.61 relative to BSA. As shown by the electrophoretic patterns in lanes 2–4, as the amount of DLPE increased to 15 mol % of the total phospholipid in the incubation mixture, the R_f value of the major products that formed during the incubation drastically decreased, indicating an increase in Stokes diameters of the products. Whenever the amount of DLPE in the incubation mixture exceeded 20 mol % of the total phospholipid (lanes 5–9), only apo A-I bands were observed (below BSA); no bands were detected higher in these lanes of the gel.

In a control experiment, several turbid aqueous dispersions of pure DMPC were prepared so that the final incubation mixtures still contained a DMPC:apo A-I molar ratio that decreased from 140:1 to 0:1, but the addition of the complementary amount of DLPE was omitted. As in the preceding experiment, each of the mixtures was incubated for 1 h at 30 °C; in contrast to mixtures that contained DLPE, the turbidity of each of these incubation mixtures cleared. The sizes of the products that formed during these incubations were determined by pore limit gradient gel electrophoresis as shown in Figure 1B. As the DMPC:apo A-I molar ratio in the incubation mixture decreased, the R_f value of the major band of the electrophoretic pattern increased, indicating a decrease in the Stokes diameters of the products. Therefore, based upon Figure 1, inclusion of DLPE in the incubation mixture radically altered the nature of the phospholipid–protein products, and the presence of PE at proportions greater than 20 mol % prevented formation of any lipid–protein complexes, including any preferential association with DMPC.

Since the spontaneous formation of phospholipid–apo A-I complexes has been shown to occur within a very narrow temperature interval surrounding the phase-transition temperature of the phospholipid, it was possible that an incubation temperature of 30 °C was not optimal for the formation of PE–PC–apo A-I complexes. Therefore, to test additional incubation temperatures, temperature-programmed heating was employed (Pownall et al., 1978; Swaney & Chang, 1980). Several turbid aqueous dispersions of phospholipid mixtures containing various proportions of PE were then incubated at a temperature that was increased from 20 °C to a final temperature of 36 °C at a rate of 3.4 deg/h. Pore limit gradient gel electrophoresis failed to show any products whenever the proportion of DLPE exceeded 30 mol % of the total phospholipid.

Thus, these initial experiments demonstrated that the incorporation of DLPE into phospholipid–apo A-I complexes via a spontaneous interaction between DLPE and apo A-I was minimal and could not be facilitated to any great extent by the inclusion of DMPC.

Bio-Beads Method for the Synthesis of Lipid–Apo A-I Complexes That Contain DLPE. Greater success in forming

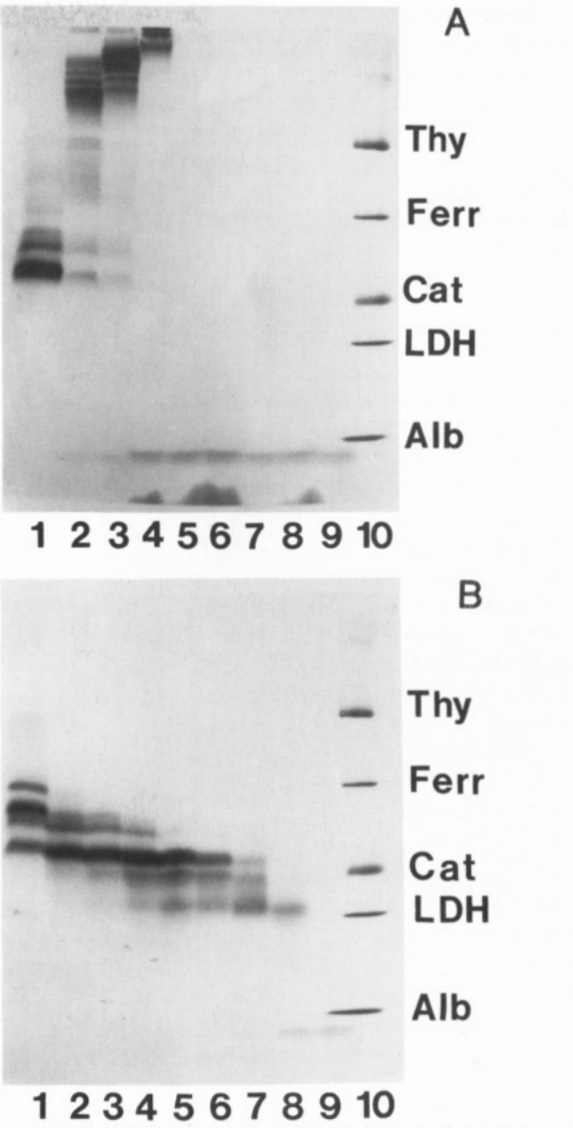

FIGURE 1: (A) Gradient gel electrophoresis of incubation mixtures that contained apo A-I and DLPE mixed with DMPC in various proportions. Aliquots of the products resulting from the incubation of aqueous dispersions of phospholipids with apo A-I (140:1 molar ratio) for 1 h at 30 °C. The proportion of DLPE mixed with DMPC in the incubation mixture was 0, 5, 10, 15, 20, 25, 50, 75, or 100 mol % of the total phospholipid (lanes 1–9). Protein standards: thyroglobulin (Thy), ferritin (Ferr), catalase (Cat), lactate dehydrogenase (LDH), and bovine serum albumin (Alb) (lane 10). (B) Gradient gel electrophoresis of incubation mixtures that contained DMPC and apo A-I in the absence of DLPE. Aliquots of the products resulting from the incubation of an aqueous dispersion of DMPC with apo A-I for 1 h at 30 °C were applied to the gel. The DMPC:apo A-I molar ratio was 140:1, 133:1, 126:1, 119:1, 112:1, 105:1, 70:1, 35:1, and 0:1 (lanes 1–9). Protein standards: thyroglobulin (Thy), ferritin (Ferr), catalase (Cat), lactate dehydrogenase (LDH), and bovine serum albumin (Alb) (lane 10).

PE-containing complexes was achieved when apo A-I was incubated at 30 °C with DLPE in the presence of cholate at a constant phospholipid:cholate:apo A-I molar ratio of 140:190:1; the phospholipid component contained various proportions of DLPE mixed with DMPC (0–100 mol % DLPE). There was complete clearing of turbidity in each case, except for the sample in which 100% of the total phospholipid was DLPE.

Bio-Beads SM-2 were added and the mixture was centrifuged to remove cholate, leaving in the supernatant fraction an eluate that contained the lipid–protein products. Chemical analysis showed that these Bio-Beads eluates contained a DMPC:DLPE molar ratio that was similar to that of the initial

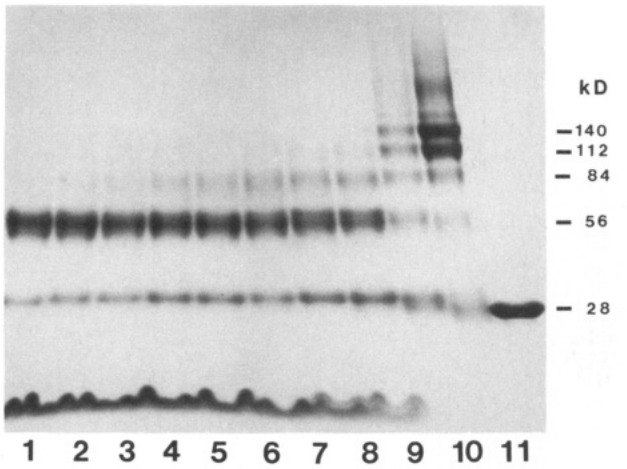

FIGURE 2: Extent of the formation and protein stoichiometry of DLPE–DMPC–apo A-I complexes prepared by the Bio-Beads method. SDS–PAGE of chemically cross-linked (+XL) Bio-Beads eluates resulting from the incubation of aqueous dispersions of phospholipid with apo A-I (140:1 molar ratio) for 1 h at 30 °C. The proportion of DLPE mixed with DMPC in the incubation mixture was 0, 5, 10, 15, 20, 25, 50, 75, or 100 mol % of the total phospholipid (lanes 1–9); lipid-free apo A-I + XL (lane 10); and lipid-free apo A-I − XL (lane 11).

incubation mixture in each case. Total phospholipid and protein recoveries in the eluate were 70–80% and 85–95%, respectively. The extent of formation of phospholipid–apo A-I complexes and the stoichiometry of the protein in these complexes were determined by chemical cross-linking the apo A-I in each of the samples (Figure 2). The number of apo A-I chains per particle in the phospholipid–apo A-I complexes was determined by reference to the electrophoretic pattern of the chemically cross-linked self-associated forms of lipid-free apo A-I, which predominantly range from monomers to pentamers with some higher order oligomers (lane 10). Whenever the proportion of DLPE was below 75 mol % of the total phospholipid in the incubation mixture (lanes 1–8), the apo A-I was predominantly cross-linked in a dimeric form, thus indicating two molecules of apo A-I were present on each particle. The electrophoretic pattern in lane 9 resembles that of the cross-linked lipid-free apo A-I (lane 10). This indicated that the Bio-Beads method could not facilitate the formation of an apo A-I complex containing exclusively DLPE as the lipid component.

Pore limit gradient gel electrophoresis was employed to assess the size of the products of each of the Bio-Beads eluates as shown in Figure 3A. As the proportion of DLPE increased from 0 to 75 mol % of the total phospholipid in the incubation mixture, the migration distance of the major product band increased, indicating that the Stokes diameters of the products decreased. Also, the heterogeneity of the products was found to decrease as the proportion of DLPE increased. A band (or series of bands) that migrates similarly to apo A-I was observed whenever PE exceeded 75 mol % of the total phospholipid, supporting our belief that complexes are not formed readily under these conditions. This band migrates similarly to lipid-free apo A-I and, as anticipated from the SDS–PAGE results, was the major band whenever the proportion of DLPE was 100 mol % of the phospholipid in the incubation mixture (lane 9).

In a control experiment, the Bio-Beads method was used to prepare lipid–protein complexes from incubation mixtures that contained DMPC:apo A-I molar ratios that decreased from 140:1 to 0:1, but from which the addition of the complementary amount of DLPE was omitted. In this experiment,

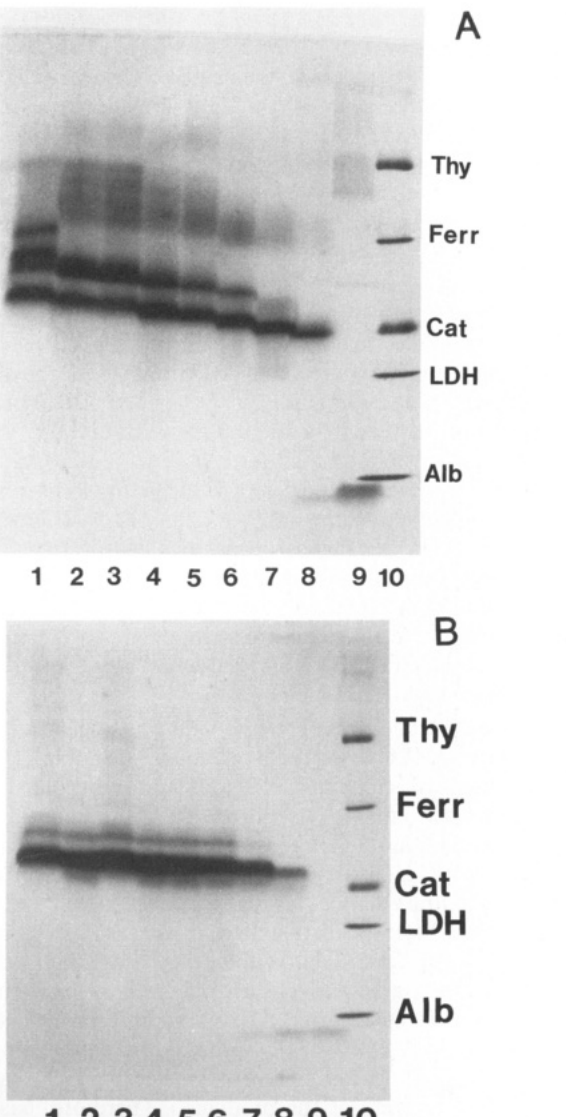

FIGURE 3: (A) Gradient gel electrophoresis of DLPE–DMPC–apo A-I complexes prepared by the Bio-Beads method. Aliquots of the Bio-Beads eluates resulting from the incubation of aqueous dispersions of phospholipid with apo A-I (140:1 molar ratio) for 1 h at 30 °C were applied to the gel. The proportion of DLPE mixed with DMPC in the incubation mixture was 0, 5, 10, 15, 20, 25, 50, 75, or 100 mol % of the total phospholipid (lanes 1–9). Protein standards: thyroglobulin (Thy), ferritin (Ferr), catalase (Cat), lactate dehydrogenase (LDH), and bovine serum albumin (Alb) (lane 10). (B) Gradient gel electrophoresis of DMPC–apo A-I complexes prepared by the Bio-Beads method in the absence of DLPE. Aliquots of the Bio-Beads eluates resulting from the incubation of apo A-I with an aqueous dispersion of DMPC for 1 h at 30 °C were applied to the gel. The DMPC:apo A-I molar ratio in the incubation mixture was 140:1, 133:1, 126:1, 119:1, 112:1, 105:1, 70:1, 35:1, and 0:1 (lanes 1–9, respectively). Protein standards: thyroglobulin (Thy), ferritin (Ferr), catalase (Cat), lactate dehydrogenase (LDH), and bovine serum albumin (Alb) (lane 10).

aliquots of the eluates were subjected to pore limit gradient gel electrophoresis (Figure 3B). In contrast to the results found whenever DLPE was present (Figure 3A), the migration distance of the major band increased only slightly as the DMPC:apo A-I molar ratio decreased, indicating that the size of this product decreased slightly. Interestingly, the sizes of the DMPC–apo A-I complexes prepared by the Bio-Beads method were nearly uniform as opposed to those prepared in the absence of cholate (Figure 1B), even though there is a large change in the initial DMPC:apo A-I molar ratio. Gels of the control samples (Figures 1B and 3B) suggest that at low lip-

FIGURE 4: Electron microscopy of DMPC–apo A-I and DLPE–DMPC–apo A-I complexes prepared by the Bio-Beads method. Enlargement of the photograph is 264 000 diameters.

id:protein ratios complexes formed by spontaneous reaction may include smaller components than those prepared from cholate dispersions, although the reason for this is not known at this time.

On the basis of the relative homogeneity of the product obtained from initial codispersions that had a DLPE:DMPC molar ratio of 1:1, shown in lane 7 of Figure 3 (R_f = 0.63), and on the high yield of this product, a PE:PC molar ratio of 1:1 was used for all PE–PC binary mixtures throughout the remainder of the study.

It was our concern to establish that the products measured in the Bio-Beads eluates contained exclusively protein–lipid complexes with no unreacted lipid or protein; several lines of evidence indicated that this was so. First, with incubation mixtures of lipid and cholate, but no protein, quantitative analysis showed that no lipid was present in the Bio-Beads eluates after the described procedure was performed. Second, when the Bio-Beads eluates from incubation mixtures containing DLPE, DMPC, and apo A-I were subjected to gel filtration on a Superose 6B column (Pharmacia, 10 mm × 30 cm column) the lipid and protein coeluted. Third, centrifugation of such an eluate on a density gradient showed coelution of protein, PC, and PE. For these reasons, the Bio-Beads eluates were used for analysis without further purification.

Electron Microscopic Studies of Lipid–Protein Complexes. The morphology of the DLPE–DMPC–apo A-I (140:140:2) reaction product was determined by negative-stain electron microscopy (Forte & Nordhausen, 1986) and compared to the morphology of particles prepared by the Bio-Beads method from a codispersion that contained a DMPC:apo A-I molar

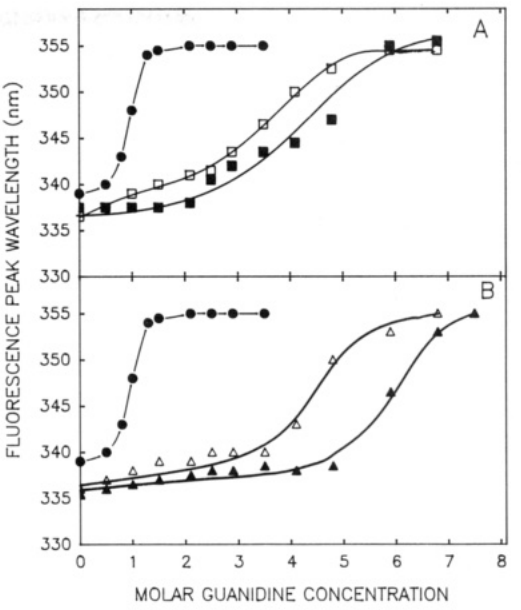

FIGURE 5: Fluorescence determination of the resistance of phospholipid–A-I complexes to denaturation. (A) Lipid-free apo A-I (●), DMPC–apo A-I complex (■), and DLPE–DMPC–apo A-I complex (□); (B) lipid-free apo A-I (●), DPPC–apo A-I complex (▲), and DLPE–DPPC–apo A-I complex (△). The points shown are the average of two separate experiments.

ratio of 140:1 without DLPE. A "two-drop" staining technique was employed; briefly, one drop of sample (100 μg of protein/mL) was applied to the grid followed by a second drop containing 1% phosphotungstic acid. Figure 4 shows the electron micrograph of DLPE–DMPC–apo A-I complexes. Rouleaux structures were observed consisting of stacked disks that were 4.5 nm thick, approximately the expected thickness of a bilayer of phospholipid. The mean diameter and standard deviation of the stacked discoidal particles was 11.4 ± 1.02 nm. Some spherical clusters of particles were observed.

Density Gradient Ultracentrifugation of Lipid–Apo A-I Complexes. The buoyant density of the DLPE–DMPC–apo A-I particle was determined by KBr density gradient ultracentrifugation (Kelley & Kruski, 1986) to be 1.12 g/mL; this is similar to plasma HDL yet more dense than DMPC–apo A-I particles prepared in the same manner, which had a density of 1.09 g/mL. Similarly, a DLPE–DPPC–apo A-I particle, prepared in a similar fashion, which was obtained from an initial codispersion with a PE:PC molar ratio of 1:1, had a density of 1.12 g/mL, in contrast to the density of the control DPPC–apo A-I particle, which was 1.10 g/mL (Bonomo & Swaney, 1988).

Stability of DLPE-Containing Lipid–Apo A-I Complexes. In order to evaluate the relative stability of the DLPE–DMPC–apo A-I and DLPE–DPPC–apo A-I particles, fluorescence spectroscopy (Verdery & Nichols, 1974) was employed. On the basis that structural changes caused by the denaturation of proteins produce changes in fluorescence emission wavelength, the resistance of apo A-I to denaturation by guanidine hydrochloride was evaluated by plotting the tryptophan fluorescence emission wavelength obtained at various guanidine hydrochloride molar concentrations (Figure 5). These changes in emission wavelength are presumed to result from local disruption of protein structure in the environment of tryptophan residues, probably accompanied by dissociation of lipid from protein, causing exposure of the aromatic side chain to the increased dielectric environment of the aqueous phase. Apo A-I was denatured at relatively low concentrations of guanidine hydrochloride. These results

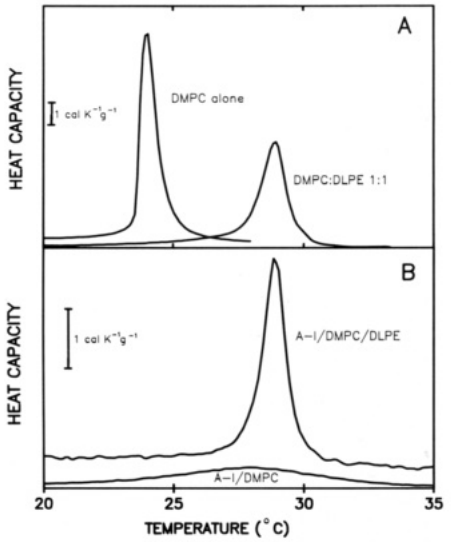

FIGURE 6: Differential scanning calorimetry of phospholipid vesicles alone (A) and phospholipid–apo A-I complexes (B). Multilamellar vesicles were prepared from DMPC only or from a 1:1 (mol:mol) mixture of DMPC and DLPE. Complexes of A-I–DMPC were prepared by the Bio-Beads technique from a mixture of 1:140 (mol:mol), while complexes of A-I–DMPC–DLPE were prepared from a mixture of 1:70:70 (mol:mol:mol).

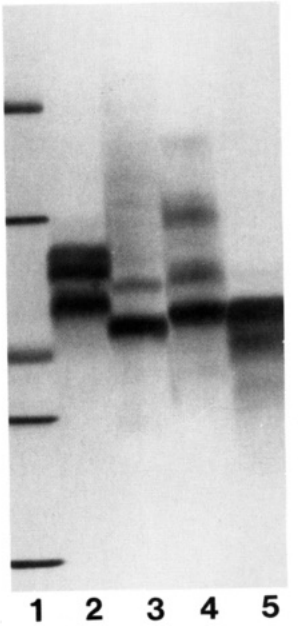

FIGURE 7: Gradient gel electrophoresis of phospholipid–apo A-I complexes prepared with various PE. Aliquots of the Bio-Beads eluates from incubation mixtures containing only DMPC or DMPC and various PE with a PE:PC molar ratio of 1:1. The protein standards are thyroglobulin (Thy), ferritin (Ferr), catalase (Cat), lactate dehydrogenase (LDH), and bovine serum albumin (Alb) (lane 1). The incubation mixtures contained components in the following phospholipid:apo A-I ratios: DMPC:apo A-I, 140:1 (lane 2); PE:DMPC:apo A-I ratios of 70:70:1 where the PE was DLPE (lane 3), DMPE (lane 4), or DPPE (lane 5).

are in excellent agreement with data previously described (Swaney, 1983; Verdery & Nichols, 1974). The phospholipid–apo A-I complexes, were, in contrast, quite resistant to denaturation, requiring a 6–8 M guanidine hydrochloride concentration to expose completely the tryptophan residues to the solvent. The DLPE-containing particles were more susceptible to guanidine hydrochloride denaturation, as contrasted with particles lacking DLPE, presumably reflecting reduced hydrophobic interaction and decreased stabilization by DLPE.

Circular Dichroism of Phospholipid–Apo A-I Complexes. To evaluate the effect of inclusion of PE on the secondary structure of apo A-I in discoidal complexes, the circular dichroic spectra over the interval 260–195 nm were obtained for DMPC–A-I (140:1 mol/mol) and DMPC–DLPE–A-I (70:70:1) complexes. The spectra for these two types of complexes appeared identical and the percentage of helix, as calculated from both the ellipticity at 222 nm and at 208 nm was 64.0% for DMPC–A-I and 62.6% for DMPC–DLPE–A-I.

Scanning Calorimetry of Phospholipid–Apo A-I Complexes. In order to further elucidate the effect of phosphatidylethanolamine on interactions within protein–lipid complexes, differential scanning calorimetry was performed on multilamellar vesicles of phospholipid alone (Figure 6A) and on complexes of apo A-I with DMPC or with DMPC–DLPE (Figure 6B). Study of multilamellar vesicles containing DMPC alone yielded a sharp endothermic transition with a midpoint at 24.05 °C and an enthalpy of 5.1 kcal/mol, which compares favorably with reported values of 23.8 °C and 5.4 kcal/mol reported by Huang et al. (Huang et al., 1989). Vesicles prepared from a 1:1 mixture of DMPC and DLPE yielded a somewhat smaller endotherm (3.2 kcal/mol) at a transition temperature of 28.95 °C. Results with complexes of apo A-I and DMPC revealed a broad transition with a T_m of 27.8 ± 0.2 °C (Figure 6B). These data are similar to those of Surewicz et al., which likewise showed a broadened transition, relative to DMPC alone (Surewicz et al., 1986). We were surprised, however, to find that the enthalpic transition with A-I–DMPC–DLPE complexes, which occurred at 28.7 ± 0.6 °C was much sharper. This suggests that the lipids in these particles are much less constrained by interactions with the A-I protein and engage in a much more cooperative melting at the phase transition (Mabrey & Sturtevant, 1976). Similar results were obtained with complexes obtained directly after elution from the Bio-Beads minicolumns and with complexes that were purified by gel filtration.

Chemical Composition of Phospholipid–Apo A-I Complexes Containing Various Species of PE. To evaluate whether this approach could be employed to incorporate species of PE other than DLPE, similar samples were prepared in which DMPE or DPPE was substituted for DLPE in a 1:1 mixture with DMPC prior to incubation with apo A-I. Samples of the Bio-Beads eluates were subjected to gradient gel electrophoresis and the results are shown in Figure 6. It can be seen that with each PE a product was obtained that generated a major band with an R_f value of about 0.62 and that was relatively less heterogeneous than the product with DMPC alone. Analysis of the chemical composition of these products is shown in Table I. As seen in Figure 7 (lanes 2–4), the distances migrated by the major band of the samples prepared with various PE, under the same conditions, were similar; however, the tabulated molar ratios indicate that while a considerable amount of DLPE or DMPE can be incorporated into phospholipid–apo A-I complexes, little, if any, incorporation of DPPE could be achieved. (Although not shown in the table, the molar ratios of the components of DPPC–apo A-I and DLPE–DPPC–apo A-I particles were approximately 170:1 and 76:69:1, respectively.)

Discussion

Although there is considerable interest in the phosphatidylethanolamine class of phospholipids, little is presently known about the impact of the presence of this phospholipid in membranous bilayers on interactions with proteins. Our ongoing interest in the interaction of apolipoproteins with phospholipids led us on theoretical grounds to study PE in this

Table I: Electrophoretic Migration and Chemical Composition of Lipid–Protein Complexes

lipid–protein complex	GGE R_f value[a] major bands	minor bands	molar ratio[b] DMPC	PE	A-I
DMPC–A-I	0.49 0.59 0.53	0.45	158 ± 18.8	0	1
DLPE–DMPC–A-I	0.63	0.45	76.4 ± 10.7	55.8 ± 11.2	1
DMPE–DMPC–A-I	0.61	0.46	89.7 ± 25.8	67.8 ± 27.6	1
DPPE–DMPC–A-I	0.62	0.68	80.0 ± 14.4	15.0 ± 16.2	1

[a] The R_f values were calculated as the electrophoretic migration distance of the band divided by electrophoretic migration distance of albumin (Figure 6). [b] Molar ratio in Bio-Beads eluate relative to apo A-I (mean ± standard deviation of four to six experiments).

context, but this concern was heightened on practical grounds with the recognition that various lipoprotein species appear enriched in PE (Banerjee & Redman, 1983; Howell & Palade, 1982; Fielding & Fielding, 1986).

Previous studies have shown that the detergent-like properties of the apolipoproteins enable them to insert in phospholipid bilayers, causing massive disruption of the bilayer and the formation of small lipid–protein complexes, but only near the phase transition temperature of the phospholipid and only with phospholipids possessing highly cooperative phase transitions (Swaney & Chang, 1980; Swaney, 1980). For these studies we selected a species of phosphatidylethanolamine, DLPE, that appeared to have the greatest likelihood of facilitating such interactions, on the basis of its phase-transition temperature. We have found, however, that under no circumstances have we been able to form complexes between pure PE (including DLPE, DMPE, or DPPE) and apo A-I, even by utilizing detergents to promote complex formation.

When we examined the spontaneous formation of complexes at temperatures around the phase transition, we found that the presence of proportionately small amounts of DLPE in a bilayer containing predominantly DMPC prevented the formation of the complexes seen usually with PC alone (Figure 1). At low proportions of PE, larger complexes were observed, but the extent of complex formation rapidly decreased so that essentially no complexes were formed when DLPE comprised more than 20% of the lipid. Although we are unaware of other studies on mixtures of DLPE and DMPC, studies on mixtures of DMPC and DMPE have indicated some immiscibility at low proportions of DMPE in the gel phase, but no immiscibility seemed apparent in the liquid-crystalline state (Chapman et al., 1974; Lentz & Litman, 1978). If, at low contents of PE, the different phospholipids segregated into separate domains one might expect at least to see complexes with DMPC comparable to those seen in the absence of PE. The fact that we observe no complexes formed like those seen in control incubations with equivalent amounts of DMPC alone (Figure 1B) implies that we are not observing a simple immiscibility of these phospholipids but that DLPE is profoundly altering the bilayer properties of DMPC.

In order to study the effect of PE on synthetic lipoproteins, we resorted to modifying a recently devised procedure, described for the synthesis of phosphatidylcholine–apo A-I complexes, which involves cholate-mediated codispersion of protein and lipid, followed by the chromatographic removal of cholate (Bonomo & Swaney, 1988). This method allowed us to prepare complexes containing high proportions of PE and might prove to be helpful in studying other types of lipid–protein interactions, such as those between membrane lipids and structural proteins, enzymes, ion channel proteins, or receptors.

The data presented here clearly establish the feasibility of the incorporation of PE into discoidal lipid–protein complexes that are similar to HDL. DLPE was found to affect the size and density of complexes; i.e., the greater the proportion of DLPE mixed with DMPC in the reactants, the smaller the size and greater the density of the resultant complex (Figures 2 and 3). This finding seems to agree with the observation that the partial specific volume of egg yolk PE is less than that of egg yolk PC (Litman, 1973).

We could not prepare a phospholipid–apo A-I complex with pure DLPE nor with a mixture consisting of greater than about 0.75 mol fraction of PE mixed with PC. This finding was similar to that of Litman (1973) who encountered problems with incorporating high proportions of egg yolk PE into egg yolk PC vesicles. Since we were unable to prepare complexes of apo A-I with pure PE even in the presence of detergent, it may be that such complexes form but are highly unstable, perhaps due to the tendency of this phospholipid to form nonbilayer structures that are incompatible with the amphipathic structure of the apolipoprotein.

The interactions of zwitterionic lipids with apoproteins are thought to be governed by hydrophobic forces (Windler & Havel, 1985). The degree of the interaction may differ depending on the chemical properties of the acyl chains and polar headgroups of the phospholipids. It is of interest that the complexes containing DLPE were less stable to denaturation by guanidine hydrochloride than those without DLPE, suggesting diminished hydrophobic interactions between the apoprotein and lipids. This might reflect variation in the nature of the lipid packing and/or in the conformation of the apolipoprotein at the interface between the protein and lipid components. Analysis of the secondary structure by circular dichroism demonstrated that there were no significant differences in the degree of α-helix between these two complexes, suggesting that protein secondary structure may not be a sensitive indicator of differences in protein–lipid interactions. This is supported by the studies of Surewicz et al., who found that the mechanism of interaction of apo A-I in complexes with dimyristoylphosphatidylglycerol was much different from that in A-I–DMPC complexes, although the protein secondary structure appeared identical in these two types of complex, as revealed by circular dichroism (Surewicz et al., 1986).

Support for these ideas comes from our data on differential scanning calorimetry of these complexes (Figure 6), which demonstrated a profound difference in the nature of the phase transition between A-I–DMPC and A-I–DMPC–DLPE. We interpret the sharper, more cooperative transition of the lipids in the PE-containing complex as reflecting a diminished interaction between protein and lipids in this type of complex. Although this does not affect the degree of helix formation in the A-I of these particles, it does appear to be manifested in a lower concentration of denaturant required to cause protein unfolding, as shown in Figure 5. This concept is also reinforced by the observation that when DMPC alone is complexed with A-I the phase-transition temperature is raised by about 3 deg (Tall et al., 1977; Surewicz et al., 1986), but complexation of a DMPC–DLPE mixture with A-I has no effect of the phase-transition temperature (Figure 6), suggesting that the protein has a minimal effect at immobilizing

the acyl chains of the phospholipids in these complexes.

Such structural variations could alter the interaction of the particles with enzymes of lipid metabolism, cell membranes, or lipoproteins or apolipoproteins. For example, Tong and Kuksis (1986) found that PE-containing triacylglycerol emulsions used in in vivo studies bound less apoprotein than those particles devoid of PE. They observed that this decrease in protein binding due to PE content seemed to result in increased catabolism of such particles relative to that of the particles devoid of PE and they hypothesized that this finding might be correlated to an increased intermolecular association observed for PE. It is of interest to note that a VLDL fraction rich in PE was also deficient in apo E (Fielding & Fielding, 1986), which again suggests that PE must modify the surface properties of lipoproteins.

In order to prepare a well-defined model lipoprotein complex, we not only analyzed the composition of the complexes but also estimated the degree of heterogeneity of the complexes. According to analysis by both pore limit gradient gel electrophoresis and electron microscopy, the DLPE–DMPC–apo A-I particles appeared to be less heterogeneous with regard to size than the DMPC–apo A-I particles. This is in contrast to work with PE-containing vesicles where it was found that the heterogeneity of the PE–PC vesicles increased 10-fold upon increasing the mole fraction of PE mixed with PE from 0.1 to 0.5 (Litman, 1973). Perhaps with our method of preparing the lipid–protein complexes the initial interaction of the bile salt with the phospholipid dispersions and subsequent addition of apoprotein dictate a certain particle size for the complexes. Electron micrographs of the DLPE–DMPC–apo A-I particles showed rouleaux structures indicative of discoidal morphology analogous to the morphology of the putative nascent HDL that accumulate due to LCAT deficiency (Forte et al., 1971).

Although information about the mechanism by which lipoproteins are assembled in vivo is scarce, it is tempting to speculate that bile acids could facilitate the formation of lipid–apolipoprotein complexes in the hepatocyte. For example, it is possible that bile acids, such as cholate and deoxycholate, following their synthesis in the hepatocyte, might associate with newly synthesized phospholipids or phospholipids donated by intracellular or plasma membranes to form phospholipid–bile acid micelles. Such bile acid–phospholipid micelles might then interact with newly synthesized intracellular apolipoproteins to form phospholipid–bile acid–apolipoprotein mixed micelles or pseudomicelles, which might be secreted with or without the bile salt component. Any elevation in PE content of these products would likely be independent of the involvement of bile acids but could reflect the elevated content of PE in certain membrane fractions (Nunez & Swaney, 1984; Valtersson et al., 1986). in this light it is interesting to note recent findings which indicate that of the approximately 33% of postprandial serum bile acids that are bound to lipoproteins, nearly 55% are bound to HDL (Hedenborg et al., 1988). The possibility exists that some of these structures result from direct secretion rather than association of resorbed bile salts with lipoprotein, but in any event this demonstrates the affinity of HDL molecules for bile acids.

This study indicates that although we were unable to form complexes containing apo A-I and pure PE, by using a detergent-facilitated methodology, considerable amounts of DLPE can be incorporated into phospholipid–apo A-I complexes with chemical and physical properties similar to nascent HDL but somewhat different from those observed with apo A-I and phosphatidylcholines alone. The ability to incorporate PE into such particles will open new avenues for studies of these particles by providing reactive amino groups, which can be used for chemical modifications, such as cross-linking of this lipid to adjacent protein domains.

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Probing the Transglutaminase-Mediated, Posttranslational Modification of Proteins during Development†

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ABSTRACT: *Sphaerechinus granularis* eggs were fertilized in seawater in the presence of 0.2 mM dansylcadaverine, and development was allowed to take place with this compound in the medium. γ-Glutamyldansylcadaverine, indicative of the utilization of the amine tracer by intrinsic transglutaminase, was isolated from the embryonic proteins, and identity of the product with the chemically synthesized γ-glutamyl derivative of dansylcadaverine was confirmed. Covalent labeling of proteins occurring during development was examined by means of electrophoresis in $NaDodSO_4$, followed by immunoblotting with an antibody that specifically recognized the dansyl hapten. There was an increase in the total uptake of the tracer at an essentially constant rate with each cell divison, from 2- to 8- and 64-cell stages. Moreover, multiple protein labeling was evident in all specimens. The described concept of studying posttranslational modifications in vivo by transglutaminase through detection of the haptenic or specific ligand recognizable group of an incorporated small amine substrate will undoubtedly be of general utility for probing the functions of this family of enzymes in other cell types as well.

Activation of transglutaminase (Lorand & Conrad, 1984), as measured by the incorporation of the naturally occurring polyamine, putrescine, into proteins, was shown to occur within a few minutes following fertilization of sea urchin eggs, suggesting that this enzyme might play a role in the early posttranslational modification of embryonic proteins (Cariello et al., 1984). An appreciable degree of protein cross-linking by N^{ϵ}-(γ-glutamyl)lysine side-chain bridges, yet another indicator of the physiological functioning of transglutaminases, was also demonstrated. These observations serve as the background for the present study which examines in vivo patterns of amine incorporation into proteins in the 2-, 8-, and 64-cell embryo. Because natural polyamines can undergo a variety of metabolic reactions, synthetic dansylcadaverine[1] was employed as the tracer (Lorand et al., 1968). This compound is a good general substrate for transglutaminases of all types and is known to compete effectively against the incorporation of polyamines into proteins following fertilization. The availability of a

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[1] Abbreviations: dansylcadaverine or Dc, *N*-(5-aminopentyl)-5-(dimethylamino)-1-naphthalenesulfonamide; γ-glutamyldansylcadaverine or γ-GluDc, N^1-L-(γ-glutamyl)-N^5-[5-(dimethylamino)-1-naphthalenesulfonyl]diaminopentane; Dns, dansyl or 5-(dimethylamino)-1-naphthalenesulfonyl; cadaverine, 1,5-diaminopentane: TLC, thin-layer chromatography; HPLC, high-pressure liquid chromatography; Boc, *tert*-butyloxycarbonyl; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetate; $NaDodSO_4$, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; K, 10^3.

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