Structure of the Major Complex Formed by Interaction of Phosphatidylcholine Bilamellar Vesicles and Apolipoprotein-Alanine (APO-C-III)†

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ABSTRACT: Apolipoprotein-alanine (ApoLP-Ala) from human plasma very low density lipoproteins has been shown previously to interact with phosphatidylcholine (PC) as determined by ultracentrifugal flotation, circular dichroism, and intrinsic tryptophan fluorescence. This protein also causes spherical bilamellar vesicles of phosphatidylcholine to become aligned as linear stacks or rouleaux when negatively stained and viewed by electron microscopy. The complex formed by interaction of PC vesicles and ApoLP-Ala *in solution* has been studied further by analytical gel filtration, analytical ultracentrifugation, and quasi-elastic light scattering. The complex eluted from a Sepharose 6B column at approximately the same volume as PC vesicles alone, but well ahead of ApoLP-Ala. Upon titration of PC vesicles with the apoprotein, the observed sedimentation coefficient increased from 1.19 S to a limiting value of 4.93 S, which was first reached when the protein-lipid ratio was 0.23 g/g. This weight ratio corresponds to a molar ratio of about 1:53 or about 46 ApoLP-Ala molecules/PC vesicle. As determined by light scattering, the average translational diffusion coefficient for a vesicle-apoprotein complex of this stoichiometry was $D_{20,w} = (2.08 \pm 0.03) \times 10^{-7}$ cm^2 sec^{-1}. These data provide direct evidence that ApoLP-Ala and PC vesicles form stable complexes which do not exist in solution as large multivesicular aggregates. In addition, the hydrodynamic data lead to the conclusions that both the hydration shell around the vesicle as well as the volume occupied by the nonpolar portion of the bilayer were changed upon complex formation with apoLP-Ala. This is consistent with a model where the apolipoprotein is bound to hydrophobic sites within the vesicle, with parts of the protein protruding into the polar head region so that interactions with hydrophilic sites on the surface of the bilayer can occur.

A recent report from this (Hoff *et al.*, 1973) and another (Forte *et al.*, 1974) laboratory have indicated that the interaction of PC[1] and ApoLP-Ala produces a lipid-protein complex which appears as a stack of discs or rouleaux when negatively stained and observed by electron microscopy (Figure 1). A similar morphology has been artificially induced by treatment of a phospholipid-cholesterol mixture with bile salts (Howell *et al.*, 1970), and by sonication of phosphatidylcholine with ApoLP-Gln-I (Forte *et al.*, 1971a). This same morphological feature has also been observed in the plasma lipoproteins of humans with biliary obstruction (Quarfordt *et al.*, 1972; Hamilton *et al.*, 1971) or with a deficiency of the enzyme lecithin-cholesterol acyltransferase (Forte *et al.*, 1971b).

While the formation of stacked discs from PC liposomes appears to be a specific effect exhibited only by proteins which strongly interact with phosphatidylcholine, it raises the important question of whether these structures actually exist *in solution*, or whether they represent the composite effects of lipid-protein interaction, negative staining, and drying.

The purpose of the present study was to characterize rigorously the structure of this complex and to establish unequivocally whether the lipid vesicles undergo apolipoprotein-induced aggregation *in solution* as suggested by the electron microscopic studies. The two components which are involved in this interaction exhibit widely differing properties. The vesicles have a molecular weight of approximately 1.86×10^6 and a partial specific volume of 0.9848 cm^3/g (Huang and Lee, 1973). The apolipoprotein contains 79 amino acid residues (Brewer *et al.*, 1972) and has a molecular weight of 9300 and a partial specific volume of 0.723 cm^3/g as calculated from the amino acid composition (Cohn and Edsall, 1943). Based on an average molecular weight of 770, there are approximately 2400 phospholipid molecules in one vesicle. Assuming a lipid-protein molar ratio of 50:1 for the complex (Morrisett *et al.*, 1973), an increase in the molecular weight of approximately 20% is expected for an individual vesicle (in addition to any further increase which might result from the formation of linear arrays or aggregates). Changes of this magnitude are easily measured by most standard techniques used to investigate the physical properties of macromolecules. In this report three different techniques were used to characterize this complex in solution: (1) gel permeation chromatography, (2) analytical ultracentrifugation, and (3) a new quasi-elastic light scattering technique, light beating spectroscopy.

Experimental Section

Preparation of Phosphatidylcholine Vesicles and Apolipoprotein-Alanine. A single buffer (100 mM NaCl-10 mM

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[1] Abbreviations used are: PC, phosphatidylcholine; ApoLP-Ala, apolipoprotein-alanine, one of the three principal C-proteins from very low density lipoproteins; ApoLP-Gln-I, apolipoprotein-glutamine-I, the major protein constituent of high density lipoproteins.

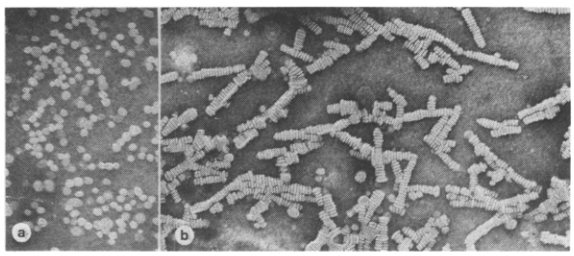


FIGURE 1: Electron micrograph of (a) PC bilamellar vesicles and (b) vesicles to which ApoLP-Ala has been added (protein/lipid = 0.25, w/w). Samples were negatively stained with 2% phosphotungstic acid (pH 7.2) as described by Hoff *et al.* (1973). Magnification, 33,333 ×. Micrograph courtesy of Dr. H. F. Hoff.

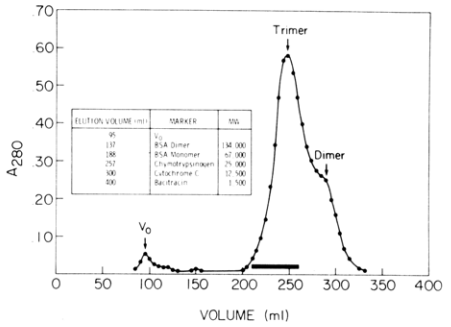


FIGURE 2: Elution profile of ApoLP-Ala (mol wt 9300). The apoprotein was chromatographed on a 2.5 × 200 cm calibrated column of Sephadex G-150 in 100 mM NaCl–10 mM Tris–1 mM NaN_3, 1 mM EDTA (pH 7.4) at 22°. The material eluting as a trimer (black bar) was pooled and concentrated by ultrafiltration for use in the analytical gel filtration, ultracentrifugation, and light scattering experiments.

Tris–1 mM NaN_3–1 mM EDTA (pH 7.4)) was used throughout these studies and is referred to hereafter as standard buffer. Phosphatidylcholine from hen's egg yolk was isolated by the method of Singleton *et al.* (1965) and was purified to homogeneity by chromatography on Unisil as described by Rouser *et al.* (1963) and stored at 5° under argon. Radioactive lecithin (used only for the gel filtration experiments) was prepared by acylation of egg lysolecithin with [^{14}C]oleic acid (New England Nuclear) as described previously (Jackson *et al.*, 1973). This material was mixed with unlabeled lecithin before sonication to give a mixture containing approximately 12,000 cpm/mg. A Bronwill Biosonik IV instrument equipped with a microtip probe was used at a power setting of 50. Phospholipid dispersions were made by sonicating 120 mg of lipid in 4 ml of standard buffer for 2 hr under nitrogen. A 17-ml Corex centrifuge tube was used to contain the dispersion and was cooled with ice–water. The temperature of the dispersion during sonication was about 15° as determined periodically with a Tri-R electronic thermometer. The phospholipid sonicate was centrifuged at 10,000*g* for 30 min to remove titanium particles and undispersed lipid. Bilamellar vesicles of uniform size (Huang, 1969) were obtained by gel filtration of the supernatant on a 1.6 × 100 cm column of Sepharose 6B equilibrated with standard buffer. Vesicles of the desired concentration were obtained either by diluting with standard buffer or by concentrating with a colloidion bag (Schlesser and Schuell) and negative pressure. Phospholipid concentration was determined by the method of Bartlett (1959).

Very low density lipoproteins were prepared from the plasma of patients with Types IV or V hyperlipoproteinemia. Apolipoprotein-alanine (ApoLP-Ala) was isolated by delipidation with chloroform–methanol (3:1), gel filtration on Sephadex G-100, and ion exchange on DEAE-cellulose as described earlier (Morrisett *et al.*, 1973). In order to minimize aggregation, the purified peptide was not lyophilized, but rather was concentrated with an Amicon ultrafilter equipped with a UM-2 membrane. The extent of self-association of the protein was determined by chromatography on a 1.6 × 200 cm column of Sephadex G-150 equilibrated with standard buffer and calibrated with bovine serum albumin (dimer = 134,000; monomer = 67,000) (Spahr and Edsall, 1964), chymotrypsinogen (25,200) (Brown and Hartley, 1966), cytochrome *c* (12,400) (Dickerson *et al.*, 1971), and bacitracin (1500) (Ressler and Kashelikar, 1966). ApoLP-Ala eluted as an unsymmetrical peak (Figure 2). The major portion of the peak was centered at 250 ml and a minor component eluted as a trailing shoulder centered at about 290 ml. These components correspond to the trimeric and dimeric forms of the molecule, respectively. The trimer-containing fractions (indicated by the solid bar in Figure 2) were pooled and concentrated by ultrafiltration to give a solution of 3.11 mg/ml. Concentration was determined by amino acid analysis and by absorbance at 280 nm (E_{280} 2.67 mg ml^{-1} cm^{-1}).

Analytical Gel Permeation Chromatography. Gel filtration experiments (Reiland, 1971) were performed using a 0.9 × 190 cm column of Sepharose 6B equilibrated with standard buffer at 4° and eluted at 8.4 ml/hr. Fractions of 1.4 ml were collected and analyzed for protein by absorbance at 280 nm and for lipid by counting ^{14}C.

Analytical Ultracentrifugation. All experiments were performed at 10° using a Beckman Model E analytical ultracentrifuge equipped with an RTIC temperature control. For sedimentation velocity experiments, the mechanical speed control was used, the refractive index gradient was observed using schlieren optics, and the results were recorded on metallographic plates. For the sedimentation equilibrium runs, the electronic speed control was used, the concentration distribution was observed using Rayleigh interference optics, and the results were recorded on II-G spectroscopic plates. Both ultracentrifuge optical tracks were focused at the $\frac{2}{3}$ plane of the cell. In all experiments, Kel F coated aluminum 12 mm double sector centerpieces and sapphire windows were used. Photographic plates were read on an LP-6 profile projector equipped with a Nikon X-Y stage, goniometer, and micrometers. The interference plate reading was performed as described elsewhere (Aune and Timasheff, 1971). The computation of molecular weight data was carried out on a Hewlett-Packard 9810 programmable calculator.

Light Beating Spectroscopy. The kinematic properties of particles in suspension have been shown to be readily assessible through measurements of the spectrum of scattered light and/or its inverse Fourier transform, the correlation function (Pecora, 1964; Pike, 1970; Cummins and Swinney, 1970). Since light scattering results from the presence of a localized fluctuation in the refractive index (*i.e.*, concentration fluctuation in a solution), a measure of the temporal decay of these fluctuations back to equilibrium can be directly related to the motion within a particle suspension.

If $i(t)$ is a measure of an instantaneous photocurrent at some time t, and $i(t + \tau)$ is its value at a later time $t + \tau$, both proportional to the intensity of the scattered light, the ensemble or infinite time average of their product is then a measure of the second-order correlation function

$$g^2(\tau) = \langle i(t) \cdot i(t + \tau) \rangle \quad (1)$$

For a monodisperse solution of scattering particles, the normalized homodyne correlation function arising from the Brownian motion of those particles can be shown to have the form

$$g^2(\tau) = 1 + e^{-2\Gamma\tau} \quad (2)$$

where Γ is the product of the translational diffusion coefficient D of the particle and the square of the scattering wave vector K. This latter parameter is related to the refractive index n, the scattering angle θ, and the reciprocal of the wavelength in vacuum λ_0, by the equation

$$K = (4\pi n/\lambda_0) \sin(\theta/2) \quad (3)$$

The measurement of the decay time constant Γ can be directly related to a value of D which in turn is defined by the Stokes-Einstein equation

$$D = kT/6\pi\eta R \quad (4)$$

In these experiments, an argon ion laser (Spectra Physics Model 165) was used as the source of the incident light. A wavelength of 4579 Å was used for these experiments at a power level of 60–100 mW. The laser beam was focused on a quartz-scattering cell (1 mm × 0.4 mm × 45 mm) containing the sample. The light was collected over a wide range of angles (22–90°) through a series of pin holes and impinged on the surface of a cooled ITT FW 130 photomultiplier (PM). The dark count of the PM tube was less than 1 count/sec at −20°. The photocurrent was then analyzed on a single clipped digital correlator which has a minimum sample time τ of 10^{-7} sec. Prior to entering the correlator, the signal from the PM tube was amplified and passed through a discriminator to shape the pulses while at the same time preserving the time displacement between them. The normalized correlation function output from this type of correlator is given by the expression

$$g^2(\tau) = 1 + f(A)\left(\frac{1 + k}{1 + \langle n\rangle}\right) e^{-2\Gamma\tau} \quad (5)$$

where $f(A)$ is the correction for spatial coherence effects (approximately equal to 0.9 for our experiments), k is the clipping level, and $\langle n\rangle$ is the average count per sample interval. Further details on clipped correlations and spatial coherence effects can be found elsewhere (Jakeman, 1970; Jakeman *et al.*, 1971a,b). A typical correlation function was measured in 10–20 sec at a clipping level $k = 0$. The data were analyzed by fitting to a single exponential between 2–3 correlation times ($\tau_c = 1/\Gamma$) using a least-squares two-parameter fit (Jakeman, 1970).

In order to obtain a measurement of the correlation time for a homogeneous dispersion of vesicles, all extraneous impurities were removed from the solution. The scattering cells were carefully cleaned, then dried with nitrogen gas. Each vesicle suspension was filtered through both 0.22- and 0.10-μ Millipore filters (cellulose acetate) directly into the scattering cell. Prior to use, the suspensions were observed under an ultramicroscope to assure that only a diffuse background was present. The concentration of the vesicles used in these studies varied between 1 and 4 mg/ml of phospholipid. Concentration of protein and lipid was determined on samples after filtration. All samples were placed in a regulated temperature chamber (±0.1°) and allowed to come to equilibrium prior to the measurement.

Results

Characterization of the Vesicle-Apoprotein Complex. GEL FILTRATION CHARACTERIZATION. The column of Sepharose 6B (fractionation range 0.1–4 × 10^6) was calibrated first with a mixture of bi- and multilamellar PC vesicles obtained by sonication. A 1-ml aliquot of the sonicate (30 mg/ml) was applied to the column and eluted at a flow rate of 8.4 ml/hr while 1.4-ml fractions were collected. Two peaks of lipid were obtained (Figure 3A). The first eluted at the void volume (43 ml) and was characterized by electron microscopy as large multilamellar vesicles (Hoff *et al.*, 1973). The second peak was centered at 57.5 ml and contained only bilamellar vesicles (1.86 × 10^6). Recovery of lipid was only 60–70% in this experiment due to nonspecific adsorption to the new Sepharose gel and column wall (Huang, 1969). Subsequent chromatography of 10.4 mg of bilamellar vesicles alone (Figure 3B) resulted in recovery of >90% of the phospholipid. To determine the elution volume of ApoLP-Ala on this column, 1.5 mg of this protein was applied and eluted under the same conditions used above for the vesicles. The protein emerged as a single peak (Figure 3C) centered at 107 ml, well away from the elution volume of either type of phospholipid vesicles.

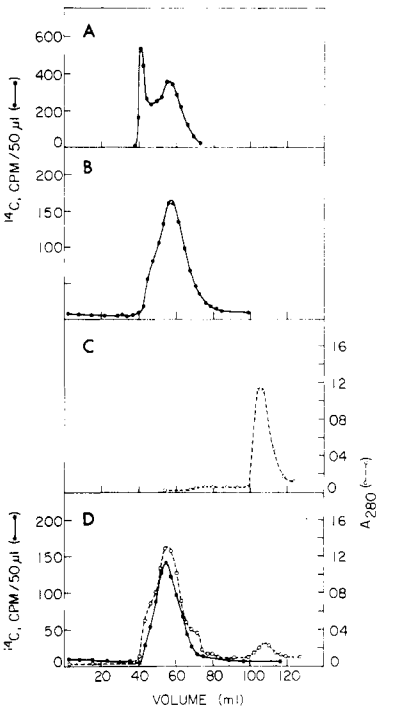


FIGURE 3: Analytical gel filtration elution profiles of (a) a sonicated dispersion of PC, (b) bilamellar PC vesicles, (c) ApoLP-Ala and (d) a mixture of ApoLP-Ala and bilamellar vesicles (0.30 g of protein/g of phospholipid) on which light scattering measurements had been made. The 0.9 × 190 cm column of Sepharose 6B was eluted at 8.4 ml/hr and 4°.

In a typical gel filtration experiment involving the ApoLP-Ala–vesicle complex, 0.38 ml of buffer, 0.30 ml of bilamellar vesicles (10.4 mg/ml), and 0.32 ml of the protein solution (3.11 mg/ml) were transferred in that order to a 5-ml test tube and stirred gently at room temperature for 2 min. The mixture (1 ml) was applied to the top of the column and eluted under exactly the same conditions as used for the protein and lipid alone. Figure 3D illustrates the chromatographic behavior of a vesicle-apoprotein mixture after analysis by light scattering. Approximately 95% of the apoprotein was eluted with the vesicle peak (55 ml). Analysis of the peak tube revealed a stoichiometry of 0.25 g of apoprotein/g of phospholipid. About 5% of the apoprotein eluted in unbound form (107 ml). The gel filtration results

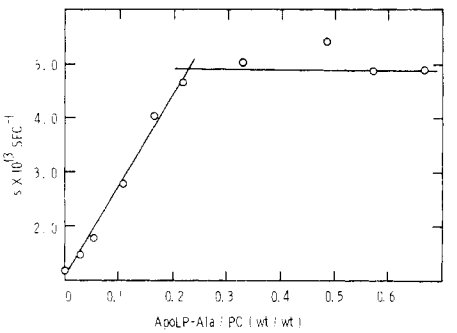


FIGURE 4: Sedimentation velocity experiment in which the observed sedimentation coefficient of ApoLP-Aa-PC vesicle complex was measured as a function of the ApoLP-Ala-phospholipid mass ratio. Conditions: temperature, 10°; solvent, 100 mM NaCl-10 mM Tris-1 mM EDTA-1 mM NaN_3 (pH 7.4); rpm, 50,740.

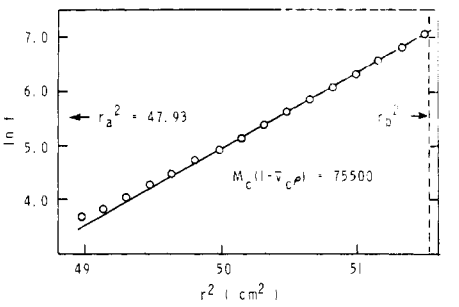


FIGURE 5: Sedimentation equilibrium experiment on the ApoLP-Ala-PC vesicle complex of 0.32 g/g from Figure 4. The natural logarithm of the fringe displacement (microns) was measured as a function of radial position squared. Conditions: temperature, 10°; solvent, 100 mM NaCl-10 mM Tris-1 mM EDTA-1 mM NaN_3 (pH 7.4); rpm, 9000; r_a is the distance of the meniscus from the axis of rotation, and r_b is the distance of the bottom of the cell from the axis of rotation.

indicate that multivesicular aggregates larger than dimers are not present in the mixture. An aggregate of only three vesicles would have a molecular weight of about 5.58×10^6, and would exceed the upper limit of the fractionation range of the gel, and hence would elute at the void volume (43 ml).

Sedimentation Velocity and Equilibrium Characterization. The two species, vesicles and protein, which are involved in complex formation are known to have quite diverse properties as mentioned earlier. The observed sedimentation coefficient of these particles is a function of the properties M, $\bar{v}$, ρ, and f in the equation

$$s = M(1 - \bar{v}\rho)/Nf \qquad (6)$$

where N is Avogadro's number, ρ is the density of the solution, and M, $\bar{v}$, and f are the molecular weight, partial specific volume, and frictional coefficient, respectively, of the sedimenting particle(s). Because the partial specific volume of a bilamellar vesicle is very near unity (Huang, 1969), the sedimentation coefficient is small in spite of the high molecular weight and small frictional coefficient (due to the spherical shape of vesicle). Thus, Huang and Lee (1973) report $s^0_{20,w}$ to have a value of 2.63 S. The present study yields a value of 2.46 S for pure vesicles at a concentration of 2.90 mg/ml, in good agreement with the former study.

The pure apolipoprotein sediments as a single component with a sedimentation coefficient, $s_{20,w}$, of 1.85 S at a concentration of 3.63 mg/ml. This value is somewhat above that expected for a compact protein of molecular weight 9300, but is consistent with the aggregation observed by gel filtration (Figure 2).

When experiments involving titration of the vesicles with ApoLP-Ala were carried out, only a single sedimenting peak was observed. But since the sedimentation coefficients of both species are of comparable magnitude, observation of a single sedimenting peak did not demonstrate unequivocally that complex formation had occurred. The results illustrated in Figure 4 show the observed sedimentation coefficient plotted *vs.* the protein-lipid mass ratio for mixtures in solutions of standard buffer at 10°. For this series of measurements, the vesicle concentration varied from 2.90 to 2.01 mg/ml. The observed sedimentation coefficient dramatically increases from 1.19 S to a limiting value of approximately 4.93 as x, the mass ratio of protein to phospholipid, increases in the range from 0.0 to about 0.30 g/g. The fact that the observed sedimentation coefficient changes implies that protein and vesicles are interacting. This change could be due to a condition where (a) the molecular weight increases, (b) the partial specific volume decreases, (c) the frictional coefficient decreases, or (d) a compensating combination of these three properties.

If the vesicles retain their original structure, complex formation implies that their molecular weight has increased. The partial specific volume of a complex formed by interaction between protein and vesicles would be expected to decrease from the value of pure vesicles toward that of pure protein if these quantities obeyed simple additivity as shown in eq 7, where $\bar{v}_c$ is the partial specific volume of the com-

$$\bar{v}_c = (x\bar{v}_p + \bar{v}_v)/(1 + x) \qquad (7)$$

plex, $\bar{v}_p$ is the partial specific volume of the protein, and $\bar{v}_v$ is the partial specific volume of the vesicles. If the complex retains the spherical shape of the original vesicle, the frictional coefficient should not change appreciably unless there were a large change in molecular weight due to aggregation of complexes. Thus, if only conditions (a) and (b) above are valid, and the binding constant for complex formation is large, then 6 and 7 may be combined to yield

$$s = (M_v/Nf)[1 - \bar{v}_v\rho + x(1 - \bar{v}_p\rho)] \qquad (8)$$

This predicts that s would increase linearly with x until the vesicles are saturated with protein. The dependence suggested in eq 8 and ensuing saturation are clearly illustrated in Figure 4. Intersection of the two lines occurs when x is equal to 0.23 g of protein/g of lipid which means that there are 53 phosphatidylcholine molecules associated with each protein molecule. This result is in good agreement with results obtained in this laboratory by other methods (Morrisett *et al.*, 1973).

If there is no association of individual complexes, the molecular weight of the predominant species in solution should be $1.86 \times 10^6 + (0.23) \times 1.86 \times 10^6$ or 2.29×10^6. A separate experiment was carried out to measure directly the molecular weight of the complex. At sedimentation equilibrium, the logarithm of the fringe displacement, ln f, should be linearly related to the square of the radial position r^2 for a homogeneous species according to the equation

$$\ln f = \ln f_a + \frac{M(1 - \bar{v}\rho)\omega^2(r^2 - r_a^2)}{2RT} \qquad (9)$$

where ω is the angular velocity, R is the gas constant, and T is the temperature. The plot of fringe displacement *vs.* r^2 in Figure 5 was obtained for an experiment where x is equal to 0.32_4 g/g. The data points clearly define a straight line and indicate the presence of a population of complexes

which is highly homogeneous with respect to molecular weight.[2] The slope of the plot leads to determination of the quantity $M_c \cdot (1 - \bar{\nu}_c \rho)$ as 75,500. Computation of $\bar{\nu}_c$ from eq 7 with x equal to 0.23 g/g yields 0.936 cm^3/g. The molecular weight of the complex, M_c, is then 1.26×10^6. This calculation rules out the possibility that the vesicles undergo protein-induced self-association *in solution* as suggested by electron micrographs of the negatively stained complex (Figure 1). However, this requires resolution of a seemingly inconsistent result since a molecular weight value for the vesicle–apoprotein complex which is lower than for the vesicle alone is not tenable with complex formation *if* the original structure of the vesicle is maintained in the complex. This apparent inconsistency is related to the partial specific volume of the complexed vesicle, $\bar{\nu}_c$. If the molecular weight of the complex is taken as 2.29×10^6 ($1.86 \times 10^6 + 0.23 \times 1.86 \times 10^6$), the data of Figure 5 allow $\bar{\nu}_c$ to assume a value of 0.962 cm^3/g. Since the gel filtration experiments indicate the size of the vesicle and complex are quite similar, then the change in $\bar{\nu}_c$ must be caused by a change in vesicle volume. Therefore, during complex formation, the partial specific volumes of the vesicle, $\bar{\nu}_v$, and apoprotein, $\bar{\nu}_p$, do not obey the simple additive relationship stated in eq 2. This aspect is treated further in the Discussion section.

Assuming that the saturated complex has a molecular weight of 2.29×10^6, a partial specific volume of 0.962 cm^3/g, and an $s_{20,w}$ of 7.59 S, the diffusion coefficient can be computed from the Svedberg equation

$$D = RTs/M(1 - \bar{\nu}\rho) \qquad (10)$$

The quantity $D_{20,w}$ was found to be 2.03×10^{-7} cm^2/sec, which is equal to that value reported by Huang and Lee (1973) for *pure vesicles*. This is also in good agreement with that value obtained in the light beating spectroscopy studies described below.

Light Beating Spectroscopy Characterization. Initially, the translational diffusion coefficient was determined for a dispersion of the bilamellar vesicles. The correlation time was measured as a function of scattering angle to determine the particle size uniformity. For a population of uniform particles, a linear relationship should exist between the decay time constant, Γ, and the square of the scattering wave vector, K. During these experiments, the vesicles were found to aggregate easily.[3] This aggregation was time and temperature dependent, and frequently was initiated by the Millipore filtration. In such cases, the samples were either refiltered or discarded. The rate of aggregation was considerably less at 10 than 20° (stable dispersion for 24 hr at 10°). Hence, the diffusion properties of the vesicles and the vesicle–protein complexes were measured at 10°. The correlation function of the vesicles decayed exponentially as seen

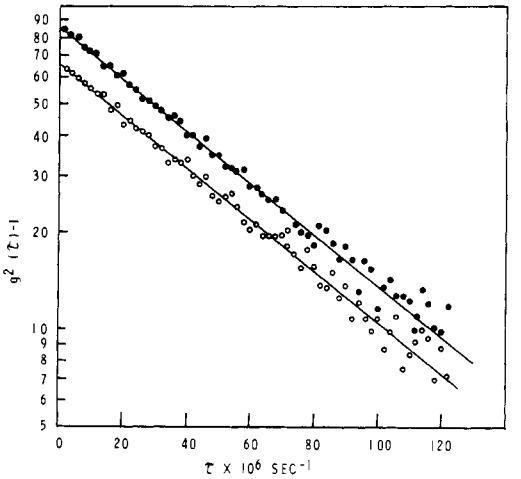


FIGURE 6: Clipped correlation function for scattered light from a suspension of phosphatidylcholine vesicles (○) and vesicles plus apolipoprotein (●) in a buffer containing 100 mM NaCl–10 mM Tris–1 mM NaN_3–1 mM EDTA (pH 7.4) at 10.3°. Least-squares fit yields a τ_c = 54.7 μsec for the vesicles and a τ_c = 55.3 μsec for the mixture corresponding to translational diffusion coefficients of $(2.09 \pm 0.11) \times 10^{-7}$ and $(2.08 \pm 0.03) \times 10^{-7}$ cm^2 sec^{-1}, respectively.

in Figure 6. The translational diffusion coefficient obtained for the phosphatidylcholine vesicles was $D_{20,w} = (2.09 \pm 0.11) \times 10^{-7}$ cm^2/sec. The dispersion was uniform as judged by the linear plot of Γ *vs.* K^2 (Figure 7A). The mean particle diameter determined from the Stokes–Einstein equation (eq 4) was 206 ± 10 Å. These values are in excellent agreement with the values previously reported by Huang and Lee (1973), who obtained an average diffusion coefficient of $(2.03 \pm 0.04) \times 10^{-7}$ cm^2/sec and a mean particle diameter of 210 ± 8 Å.

To study the lipid–protein complex, a saturation mixture of apolipoprotein and PC vesicles (0.32 g of protein/g of lipid) was prepared and incubated at 10° for about 30 min. Both components were filtered prior to the preparation of the mixture. The intensity from the apolipoprotein sample was less than 1% of that measured for the vesicle solution and was, therefore, neglected in the measurements on the complex. Correlation times were obtained for the complex over the same range of scattering angles as for the vesicles. No increase in the scattered intensity or correlation times was observed for the mixture. The correlation function from the complex also decayed exponentially (Figure 6) indicating a single component, and the decay time constant varied linearly with K^2 (Figure 7B). The average translational diffusion coefficient obtained for the mixture was $D_{20,w} = (2.08 \pm 0.03) \times 10^{-7}$ cm^2/sec and the mean particle diameter was 206 ± 4 Å. From these data, it is apparent that large multivesicular aggregates are not present in the solution mixture. Furthermore, since no change in the diffusion coefficient was observed for the complex, it appears that binding of the apoprotein did not alter significantly the hydrodynamic size of the vesicle.

Discussion

The gel-filtered and Millipore-filtered vesicles used in this study were found by quasi-elastic light scattering and sedimentation measurements to be of uniform particle size. The physical properties of these phospholipid vesicles determined from diffusion and sedimentation measurements agree well with previous results (Huang, 1969; Huang and Lee, 1973). Data obtained from measurements on the

[2] Excess uncomplexed protein of molecular weight 3 × 9300 would not contribute significantly to the fringe displacement under conditions of this experiment.

[3] The most abundant aggregated forms had the size of dimers, trimers, and tetramers and appeared as discrete particles against a diffuse background of monomeric vesicles when viewed with an ultramicroscope. The estimated abundance of these aggregates was <5%. Since the homodyne correlation function is proportional to the square of both the concentration and molecular weight of the particle, the weighted contribution of these aggregates to that function is disproportionately greater than their relative abundance. Hence, the removal of these aggregates from the monomer population before beginning a scattering experiment was necessary. The size and abundance of these aggregates were much less than those observed by electron microscopy.

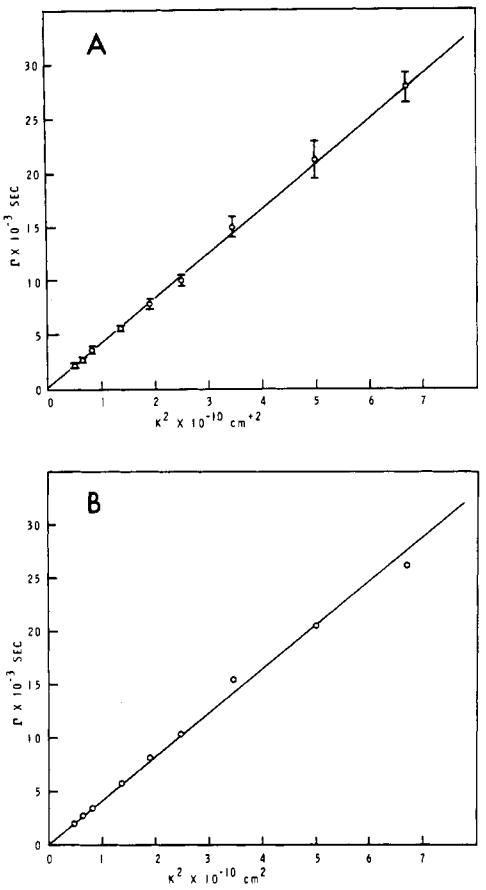
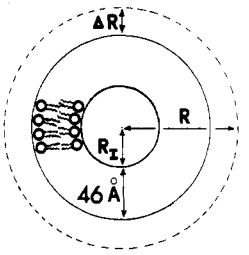


FIGURE 7: Decay time constant as a function of K^2 for a solution of (a) phosphatidylcholine vesicles, and (b) vesicles plus apoLP-Ala at a weight ratio of 0.31 g of protein/g of lipid, corrected to 20° and standard solvent, water.

major[4] complex formed by interaction of these vesicles and ApoLP-Ala indicate that *in solution,* the lipid-protein complexes do not exist as multivesicular aggregates as suggested by electron micrographs of negatively stained complexes (Figure 1, Hoff *et al.,* 1973). By inference, it is highly doubtful that a significant concentration of aggregates is present in sonicated dispersions of phosphatidylcholine and ApoLP-Ala (Forte *et al.,* 1974) or in the lipoproteins of patients with biliary obstruction (Hamilton *et al.,* 1971; Quarfordt *et al.,* 1972). Both the sedimentation velocity and gel filtration studies provided direct evidence that a lipid-protein complex was formed and that at saturation, the ApoLP-Ala/PC mass ratio was 0.23–0.25 g/g, corroborating the earlier results of Morrisett *et al.* (1973). The diffusion measurements not only demonstrated that no linear aggregates were formed, but also indicated that no detectable change in the size of the vesicles had occurred as a result of complex formation. This latter result was not too surprising since the increase in the hydrodynamic radius of the vesicles would be only approximately 5 Å if the apolipoprotein *volume* were distributed uniformly over the outer surface of the vesicle.

[4] There is evidence from preparative density gradient and gel filtration experiments (Morrisett and Gotto, 1974) that when the starting protein–lipid ratio is on the order of 0.67 (w/w), a very protein rich, lipid poor complex can form which has a molecular weight greater than ApoLP-Ala but much less than a PC vesicle. Such a complex would not have had a significant effect on the ultracentrifugal or light scattering experimental results.



$$R = \Delta R + 46\,\text{Å} + R_I = 103\,\text{Å}$$

FIGURE 8: Spherical model of a phosphatidylcholine bilamellar vesicle where R_I is the radius of the internal aqueous compartment, R is the full radius of the hydrated vesicle, and ΔR the width of the hydration shell. The width of the phospholipid bilayer is taken as 46 Å (Small, 1967).

The observed change in the sedimentation coefficient as the mass ratio of apolipoprotein to phospholipid increased from 0.0 to 0.23 g/g implied that the molecular weight and partial specific volume of the vesicle had changed during the formation of the complex. From a sedimentation equilibrium measurement performed on an apoprotein–phospholipid mixture corresponding to a point in the saturation region (Figure 4), the partial specific volume was found to decrease from 0.9848 (Huang and Lee, 1973) to 0.962 cm^3/g, rather than to the value of 0.936 cm^3/g predicted on the basis of eq 7. It was apparent from this result that a volume change in the protein, the vesicle, or both must have taken place during complex formation. Volume changes associated with conformational changes of protein are generally quite small (Katz and Denis, 1970). Hence, it is reasonable to propose that this result was due chiefly to a volume change in the lipid bilayers upon interaction with the apoprotein. A calculation of $\bar{v}_v$ from a rearranged form of eq 7 and assuming that $\bar{v}_c$ is 0.962 cm^3/g leads to a value of 1.017 cm^3/g for $\bar{v}_v$. This suggests that PC molecules undergo a volume change of +0.032 cm^3/g (0.985 + 0.032 = 1.017) when protein complexes with the vesicles. This change is about the same magnitude as the volume changes observed by dilatometry for gel → liquid crystalline transformations. Melchior and Morowitz (1972) have found volume changes of +0.023, +0.026, and +0.036 cm^3/g for dimyristoyl-, dipalmitoyl-, and distearoyl-L-α-lecithin, respectively. The egg lecithin used in this study might be expected to have comparable volume changes since it is composed of 38% palmitic, 15% stearic, 32% oleic, and 16% linoleic acids.

If such a volume change takes place, it would seem reasonable that the partial specific volume of the vesicle increases linearly for $0 < x < 0.23$ and has a value 1.017 cm^3/g when $x > 0.23$. Hence, a sedimentation equilibrium experiment carried out where x is less than 0.23 g/g should yield a molecular weight of $(1 + x)M_v$ when $\bar{v}_v$ is computed on the basis of the above assumption. In such an experiment where x was equal to 0.086 cm^3/g, the quantity $M_c(1 - \bar{v}_c\rho)$ was found to be 53,400 ± 2400. Under these conditions, $\bar{v}_v$ is calculated to be 0.997 cm^3/g and $\bar{v}_c$ is calculated to be 0.975 cm^3/g from eq 7. This leads to a value of $(2.5_5 \pm 0.3) \times 10^6$ to be compared with the predicted value of 2.02×10^6 for $x = 0.086$ g/g. In view of the assumptions made and the sensitivity of the quantity $(1 - \bar{v}_c\rho)$ to small errors in $\bar{v}_c$, these values are considered to be in agreement.

Recently, Johnson (1973) has discussed the sedimentation of a hollow lipid vesicle in terms of a sphere having dis-

continuous layers of differing densities. The same model (Figure 8) has been used in the present study to interpret the observed changes in the physical properties of PC vesicles upon addition of apolipoprotein. An estimate of the degree of hydration, δ_0, for this model can be obtained for the vesicle and its complex by combining the results from the sedimentation and diffusion data into eq 11. The fully hydrated vesicle contains an inner volume of trapped water. Recent paramagnetic resonance studies indicate that there is also a significant hydration shell on the surface of the phospholipid bilayer (Griffith *et al.*, 1974). Therefore, any calculation of the amount of hydration must include the water present in the inner volume and in an outer hydration shell. By substituting into eq 11 the appropriate values for the radius, molecular weight, and partial specific volume, the amount of water associated with the vesicle was found to be 0.47 g/g of lipid. In the case of the complex, the amount of water was found to be only 0.22 g/g of lipid, implying displacement of water from the hydration shell during apoprotein binding.

$$\delta_0 = \frac{(4\pi R^3/3) - (M\bar{v}/N)}{V_0} \qquad (11)$$

The thickness of the hydration shell of the vesicle and its apoprotein complex can be obtained by relating a bilayer volume of constant thickness to the width of the outer hydration shell. If a value of 46 Å (Small, 1967) is used for the thickness of a PC bilayer and if ΔR is the width of the hydration shell, then the volume, V_{bl}, of the bilayer (in Å^3) can be determined from

$$V_{bl} = \frac{4\pi}{3}[(103 - \Delta R)^3 - (57 - \Delta R)^3] \qquad (12)$$

The width of the outer hydration shell, ΔR, was calculated to be approximately 8.7 Å with an inner radius, R_1, of 48.3 Å for the vesicle. When the calculation was performed for the complex, it was found that the hydration shell becomes approximately 1.5 Å with the inner radius equal to 55.5 Å. These model calculations are consistent with (1) an increase in bilayer volume and (2) a decrease in surface hydration for the PC vesicle during interaction with ApoLP-Ala. Such changes suggest that the apolipoprotein is bound to hydrophobic sites within the bilayer with parts of the protein protruding into the polar region so that interactions with hydrophilic sites on the surface of the bilayer can occur. This view is entirely consistent with a recently proposed mechanism for lipid-apolipoprotein interaction based on sequence and model building studies (Segrest *et al.*, 1974).

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