Size and Shape of the Model Lipoprotein Complex Formed between Glucagon and Dimyristoylglycerophosphocholine†

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ABSTRACT: Glucagon forms water-soluble lipoprotein particles with dimyristoylglycerophosphocholine at temperatures below the phase-transition temperature of the lipid. The shape and size of this lipoprotein particle were studied by viscometry, sedimentation velocity, sedimentation equilibrium, quasi-elastic light scattering, and electron microscopy using both negative-staining and freeze-fracture techniques. The lipoprotein particle has an oblate ellipsoid shape with dimensions of 250 × 70 Å and an approximate molecular weight of 1.4×10^6. This molecular weight is similar to that found for small unilamellar phospholipid vesicles but is achieved in the presence of glucagon without sonication. The shape of the glucagon lipoprotein particle is similar to that found for the complex formed between some serum apolipoproteins and dimyristoylglycerophosphocholine. From these data, a model for the glucagon–dimyristoylglycerophosphocholine is proposed consisting of a single bilayer of phospholipid with the glucagon incorporated into the bilayer structure in such a manner as not greatly to disturb the average area occupied per phospholipid molecule.

Glucagon, a peptide capable of forming amphipathic helices, has the unusual property of interacting more strongly with lecithin when it is in the gel state than when it is in the liquid-crystal state (Epand et al., 1977a,b). The interaction results in the solubilization of lipid at temperatures below their phase transition. This paper is concerned with the shape and size of the particle which is formed between glucagon and dimyristoylglycerophosphocholine (DMPC)[1] and its resemblance to certain serum lipoprotein particles.

Materials and Methods

Materials

Commercial preparations of crystalline bovine–porcine glucagon (Elanco Corp.) and DMPC (Sigma Chemical Co.) were characterized as previously described (Epand et al., 1977a,b).

Methods

Preparation of the Glucagon–DMPC Complex. Solutions of this lipoprotein complex at a concentration of 3–4 mg/mL in 0.1 M ammonium acetate, pH 7.4, were prepared as previously described (Epand et al., 1977b). This solution was diluted with KBr for some of the ultracentrifugation analyses. The concentration of glucagon was routinely determined from its optical density at 278 nm, suitably corrected for light scattering, using $E_{1cm}^{1mg/mL} = 2.12$ (Epand et al., 1977b). However, on small or dilute samples and fractions, chemical methods were employed. Since KBr interferes with the Lowry test, density-gradient fractions were analyzed by the fluorescamine method described by Nakai et al. (1974) using a Perkin-Elmer MPF-44 fluorimeter. Protein concentrations in density-gradient experiments were alternatively monitored by using ^{125}I-labeled glucagon tracer (N.E.N.) which was directly counted in a γ counter. For samples from gel-exclusion chromatography, undissolved lipid interference was eliminated by including 0.5% $NaDodSO_4$ (Pierce, Sequanal grade) in the solutions for Lowry protein determination (Lowry et al., 1951) which was calibrated with glucagon whose concentration was determined spectrophotometrically (Gratzer et al., 1967). Lipid concentrations were determined by the method of Bartlett (1959) after perchloric acid ashing of the samples.

Determination of the Peptide–Lipid Ratio in the Complex. In the absence of lipid, the amount of glucagon solubilized at pH 7.4, 6 °C, is less than 0.01 mg/mL. Two milliliters of a 1 mg/mL suspension of glucagon in 0.1 M ammonium acetate was added at 38 °C to varying amounts of lipid, up to 10 mg, which had been deposited as a film on the walls of a glass tube. After cooling to 6 °C and centrifugation at this temperature, the supernatant was analyzed for lipid and glucagon as described above. The measured concentration of glucagon was corrected for the amount which dissolved in the absence of lipid. A constant ratio was obtained for the excess glucagon solubilized by the lipid. This ratio was not affected by varying the ionic strength between $\Gamma/2 = 0.003$ and 0.75 M. This ratio, however, is not the true stoichiometry. Storage of these solutions at 0 °C causes slow and variable precipitation of glucagon. It appears that in the presence of the freshly prepared lipoprotein complex, additional glucagon can dissolve which is not intimately associated with the lipoprotein particle. After 24–72 h at 0 °C, this extra glucagon may be removed by centrifugation (see Results for effects of "aging" and determination of true lipid–peptide ratio). High concentrations of free glucagon are known to produce β structures of increasing molecular weight and form fibrils and gels (Beaven et al., 1969); for measurement of hydrodynamic parameters which are sensitive to small amounts of high-molecular-weight material, we therefore used solutions of "aged" complex. Preliminary results have been reported (Jones et al., 1977; Jones & Epand, 1977) in which "fresh" complex was used and these results suffer from *over*estimates of the molecular weight and

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[1] Abbreviations used are: DMPC, 1,2-dimyristoyl-*sn*-3-glycerophosphocholine; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; EDTA, (ethylenedinitrilo)tetraacetic acid; OD, optical density; *C*, concentration of glucagon–DMPC complex; *r*, distance from the center of the rotor in the analytical ultracentrifuge; $NaDodSO_4$, sodium dodecyl sulfate; ρ, density; *n*, refractive index; RMS, root mean square; SEM, standard error of the mean; *a/b*, ratio of major to minor axes of ellipse; *h*, length of cylinder; *d*, diameter of cylinder.

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*under*estimates of the lipid/protein ratio as a consequence of this supersaturation phenomenon.

Intrinsic Viscosity. Reduced viscosities of the glucagon–DMPC complex were measured in Cannon-Ubbelohde semimicro dilution viscometers in a Cannon water bath maintained within ±0.002 °C at a set temperature of 20 °C. Within the concentration range used (1–3 mg/mL), no significant concentration dependence was observed. Viscometers with flow times for buffer of 138 and 283 s were used, and non-Newtonian flow was not observed. The value for the intrinsic viscosity $[\eta]$ was calculated from the mean of two preparations of complex, each of which was measured at four different concentrations.

Sedimentation/Flotation Velocity. The Svedberg equation can be rewritten in the form $\rho = (1/\bar{v}) - (RTs/DM\bar{v})$. Thus, a plot of ρ against s will produce a straight line having an intercept of $1/\bar{v}$ and a slope of $(-RT)/(DM\bar{v})$. This is essentially the same treatment for velocity data as that used for sedimentation equilibrium data by Nelson et al. (1974) for determining both $\bar{v}$ and M of serum lipoprotein by using solvents of different densities.

Solid KBr was added to samples of complex to adjust the density to the range 1.2–1.34 g/cm^3. This solution was then diluted with 10 mM Hepes, 0.1 mg/mL EDTA, 0.02 mg/mL NaN_3 (pH 7.4), and KBr to achieve the desired density. Density measurements and velocity runs were carried out at 12 °C. A Spinco Model E analytical ultracentrifuge equipped with a Beckman Dynograph UV scanner was used in conjunction with an AN-H-Ti rotor containing two double-sector cells. The reference sector contained buffer adjusted to the same density as the solution.

Five or six scans of the contents of each cell were made during each run at either 26 033 or 32 042 rpm and the time at which the boundary was scanned was plotted against log r. The plots yielded straight lines with regression coefficients in excess of 0.998. Schlieren optics were used for the sample in 0.1 M ammonium acetate, without KBr, at 20 °C. Observed values of s were corrected to the viscosity of water at 20 °C for runs made at other temperatures and KBr concentrations, before plotting ρ vs. s. Values for the viscosity of KBr solutions were obtained from the International Critical Tables and are about 10% lower than water at 12 °C. Concentrations of lipoprotein complex between 4 and 0.8 mg/mL were used for runs in the absence of KBr and a value of $s^0_{20,w} = 9.2$ S was obtained by extrapolation to infinite dilution. For the runs in the presence of KBr, the lipoprotein concentration was only 0.15 mg/mL and no attempt was made to extrapolate to infinite dilution.

Flotation Equilibrium. The method of Nelson et al. (1974) using the UV scanner was employed for measuring the equilibrium distribution of the lipoprotein complex at speeds of 3005, 3400, and 4001 rpm depending on the density of the solvent. Runs at each density were allowed to come to equilibrium at two different speeds: times of 24–36 h for the lower speed and 12–24 h after the speed was increased were found to be sufficient for the attainment of equilibrium. The contribution of the free glucagon to the equilibrium optical density (OD) values was estimated from the residual OD at the bottom of the cell after the boundary had floated up in velocity runs performed on the same samples as described above. Glucagon (molecular weight 3500, $\bar{v} = 0.72$) does not significantly distribute at these low speeds. The OD value so obtained in the velocity runs was taken as a baseline, and equilibrium distributions were calculated accordingly. No significant curvature of the plots of $d(\ln C)/dr^2$ was observed at any of the solvent densities employed.

Density Measurements. For the determination of $\bar{v}$ of the lipoprotein complex, a 25-mL pycnometer was used and equilibrations were carried out in a Cannon water bath maintained to ±0.002 °C at a set temperature of 20 °C. SEM's of 10–20 weighings each for sample, solvent, and pycnometer were less than 1 in 5×10^5. Density of water at 20 °C was taken as 0.99823_4. Concentration of solute was determined by dry-weight analysis. The densities of the KBr solutions used for the ultracentrifugation analysis were also determined with this pycnometer after equilibration at 12 °C.

Density-Gradient Ultracentrifugation. Samples of complex were adjusted to $\rho = 1.25$ with solid KBr and overlayed with a 9-mL linear gradient of KBr in 10 mM Hepes, 0.1 mg/mL EDTA, 0.2 mg/mL NaN_3 (pH 7.4), $\rho = 1.21 - 1.00$. After centrifugation in an SW 41 rotor of a Beckman Model L-2 preparative ultracentrifuge at 34 000 rpm (140 000g_{av}) for 44 h at 10 °C, the gradients were fractionated with an Isco gradient fractionator (Model 185) into 405 ± 5 μL fractions which were collected manually in Eppendorf micro centrifuge tubes. Densities were measured in calibrated 200-μL Pedersen constriction pipettes or by refractive-index measurement. Refractive-index measurements were made on a Bausch and Lomb refractometer calibrated with solutions of KBr in buffer whose densities had been accurately determined by pycnometry (see above). Phosphate content was determined by the method of Bartlett (1959) after perchloric acid ashing at 180 °C. The precipitate formed by KBr in this reaction was found not to affect the precision of the method. Protein was determined chemically as described above.

"Blanks" using lipid alone were performed on vesicles prepared by sonicating to clarity in Hepes–EDTA–NaN_3 both in the absence and in the presence of KBr and adjusted to a final density of 1.25 after sonication.

Quasi-elastic Light Scattering. In these experiments, an argon ion laser (spectra Physics Model 165) was used as the source of the incident light. A wavelength of 4880 Å was used at a power level of 800 mW. The laser beam was focused on a thermostated scattering cell containing the sample. Sample temperature was continuously monitored (±0.1 °C) by a thermocouple immersed in the upper portion of the cell. The light was collected at an angle of 90° through two pinholes and impinged on the surface of a cooled ITT 7W 130 photomultiplier (PM). The dark count of the PM tube was less than 1 count/s at −20 °C. The photocurrent was then analyzed on a single clipped digital correlator which has a minimum sample time Γ of 2.5×10^{-7} s. Prior to entering the correlator, the signal from the PM tube passed through a photon discrimination unit (Mech-Tronics Model 511). The normalized correlation function output from this type of correlator is given by the expression $g^2(\tau) = 1 + f(A)[1/(1 + n)]e^{-2|\tau}$ where $f(A)$ is the correction for spatial coherence effects (about equal to 0.85 for these experiments), and n is the average count per sample interval. A typical correlation function was measured in 3.5 s. The data were analyzed by fitting to a single exponential about two correlation times using a least-squares two-parameter fit (Jakeman, 1970). RMS deviation of the experimental data to the calculated decay was <0.6% for 2×10^5 accumulated counts for an individual run. Each sample was analyzed at least four times with resultant SEM's of ~±2%.

Refractive Index Increment. Measurements were made with a Brice Phoenix BP-2000-V differential refractometer using light of wavelength 546 nm isolated from the mercury arc lamp by an interference filter. Sample temperature was maintained at approximately 18 °C by circulating water. Five–eight measurements were made for each of the duplicate samples

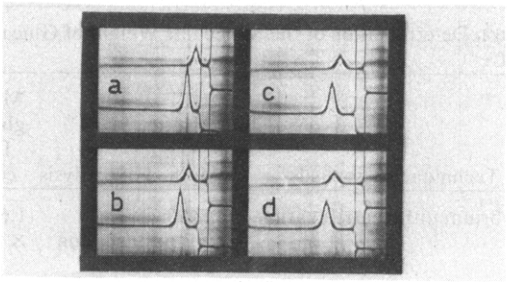

FIGURE 1: Schlieren photographs of glucagon lipoprotein in 0.1 M ammonium acetate (pH 7.4) at 12 °C. Exposures were made at (a) 4, (b) 8, (c) 12, and (d) 16 min after reaching a speed of 55 896 rpm. The upper trace concentration was 1.05 mg/mL and the lower was 2.25 mg/mL of lipoprotein complex.

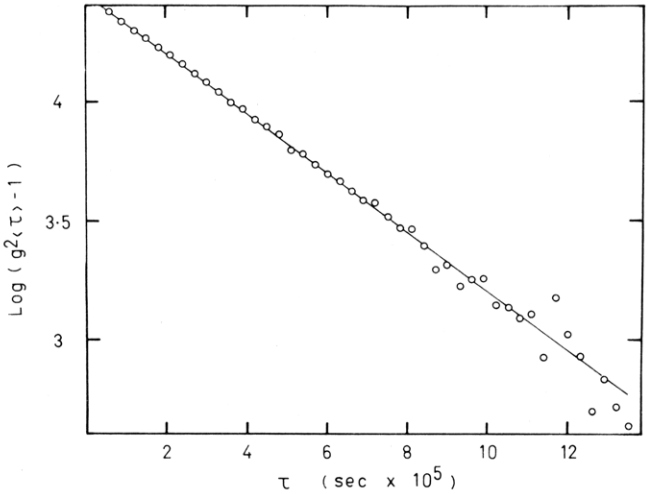

FIGURE 2: Clipped correlation function for scattered light from glucagon lipoprotein at 3.5 mg/mL in 0.1 M ammonium acetate (pH 7.4). Measured at 21 °C. Least-squares analysis yields a time constant of 68.78 μs corresponding to a translational diffusion coefficient of 2.47×10^{-7} cm^2 s^{-1} with an RMS deviation of ±0.48%.

at five different concentrations of complex. Least-squares analysis yielded a value of $dn/dC = 0.131 \pm 0.003$ mL g^{-1}.

Electron Microscopy. Solutions of the glucagon–DMPC complex in 0.1 M ammonium acetate were used for negative staining. All manipulations were done at a room temperature of 20 °C. The characteristic small, rod-shaped particles found by this procedure were not observed when the preparation was carried out in an environmental chamber at 35 °C. Freeze-fracture was done on a 4 mg/mL solution of the glucagon–DMPC complex in 0.1 M ammonium acetate.

The techniques used for sample preparation for electron microscopy are the same as previously described (Papahadjopoulos et al., 1975), except that bacitracin was used in place of bovine serum albumin to coat the grids for negative staining with ammonium molybdate, pH 7.4.

Gel Exclusion Chromatography. Sepharose 4B was washed repeatedly and suspended in 0.1 M ammonium acetate (pH 7.4) and packed into a 0.6 × 60 cm column and equilibrated at 4 °C under ~80-cm hydrostatic pressure. After passage through the column of mixed size populations of DMPC vesicles, to saturate any phospholipid binding sites (Huang, 1969) on the matrix, a sample of saturated glucagon solution was applied. This eluted in the column volume showing that, during the progress of the elution, free glucagon does not extract lipid bound to the column, i.e., does not form a complex. The column was therefore suitable for separating free glucagon from complex in order to determine the protein–lipid ratio in the absence of interference from free glucagon.

Results

Homogeneity of the Glucagon–DMPC Complex. Evidence for a narrow size distribution among particles of complex comes from a variety of physical techniques. The single peak obtained in flotation and/or sedimentation velocity experiments (see Figure 1) can be analyzed by the method of second moments (Goldberg, 1953) to detect asymmetry and nonhomegeneity. When this method is applied to the complex, it yields results which differ from the conventional treatment (using only the maximum of the peak) by less than 1%. Linear plots of ln C vs. r^2 obtained in sedimentation/flotation equilibrium experiments are also strongly suggestive of the presence of a single component. The time constant for the monoexponential decay of the autocorrelation function, determined by quasi-elastic light scattering, is used to determine the diffusion coefficient of the particle being examined. The goodness of fit of the calculated decay to the experimental decay (Figure 2) (in this case the deviations ranged from 0.2 to 0.5% RMS, see Methods section) is indicative of a narrow size range, since polydisperse systems give rise to multiexponential decays with a resultant increase RMS deviation. A direct visualization of the particles in negatively stained electron micrographs (such as those seen in Figure 6a) allows an estimation of the size distribution. With samples taken from several different micrographs dimensions of 249 ± 6 × 68 ± 2 (SEM; $n = 85$) were obtained.

Partial Specific Volume, $\bar{v}$. Ultracentrifugation analysis may accurately be interpreted only if $\bar{v}$ is known with sufficient accuracy. As $\bar{v}$ approaches 1, then small errors in $\bar{v}$ greatly affect the buoyancy term $(1 - \bar{v}\rho)$ and therefore the molecular weight determined by centrifugation techniques. We have employed several techniques to determine $\bar{v}$ (in order to increase the accuracy of the molecular weight estimates).

Density gradient centrifugation yielded a value of 0.916 ± 0.003 mL/g (SEM; $n = 5$). When DMPC vesicles were analyzed in a density gradient, they banded at $1/\rho = 0.932$ mL/g, in good agreement with the value of $1/\rho = 0.926$ mL/g reported by Pownall et al. [1974; quoted by Morrisett et al. (1977)]. These values are lower than the values of 0.972 and 0.963 mL/g at 28 and 23 °C, respectively (i.e., above and at the phase-transition temperature) reported by Aune et al. (1977). Since the density gradients were run at 6 °C, it is not possible to distinguish between the effects of temperature and high salt concentration on the value of $\bar{v}$.

The estimate of $\bar{v}$ obtained from density perturbation equilibrium flotation runs in which ρ is increased by added salt was 0.88 ± 0.02. The molecular weight determined simultaneously from these runs was $1.6 \pm 0.2 \times 10^6$. Since these measurements were made using the UV scanner, the distributions calculated are sensitive to the presence of free glucagon. This was corrected for, to a first approximation, by assuming that the free glucagon was uniformly distributed throughout the cell and was not sedimentable under the conditions used to determine the OD due to the glucagon. However, the presence of oligomers of glucagon (see Methods) could not be excluded and could account for the large error in the above values. Another possible source of error in these measurements may be a long-term instability of the complex, such as dissociation, leading to a liberation of glucagon and precipitation of the lipid it had been associated with. It should be noted, however, that the complex that remained in solution was unaltered (see below).

Because of these possible complications, we have introduced a variation of the density-perturbation technique using velocity

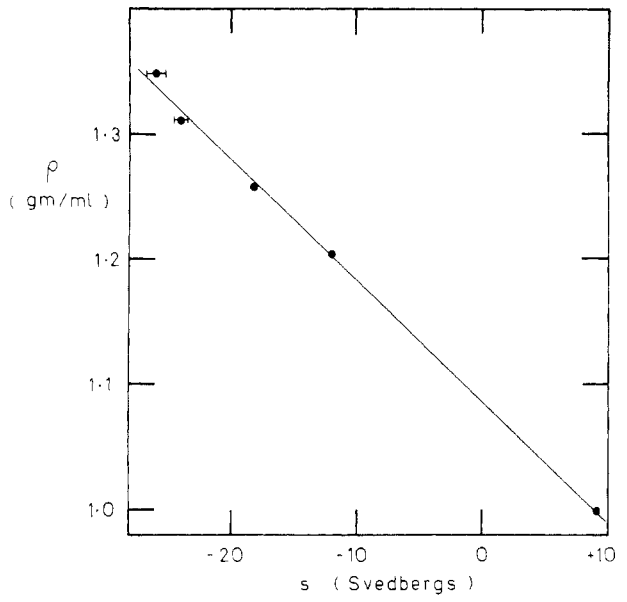

FIGURE 3: Dependence of s value upon solvent density; ρ was adjusted to the desired value with KBr. s values have been corrected for temperature and solvent viscosity to standard conditions of 20 °C in water. Least-squares analysis yields a value of $1/\rho$ at $s = 0$ of 0.919 ± 0.005. Error bars are within the symbols, except where shown.

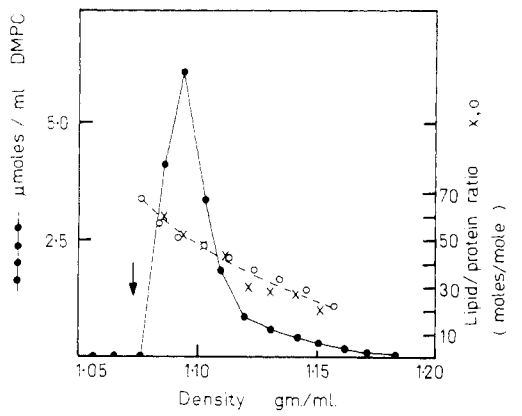

FIGURE 4: Equilibrium density gradient centrifugation of glucagon lipoprotein in 10 mM Hepes, 0.1 mg/mL EDTA, 0.2 mg/mL NaN_3 (pH 7.4) with KBr to give $\rho = 1.21 - 1.00$. Lipid (●) was detected using the method of Bartlett (1959), and protein was determined either by fluorescamine (x) or with [^{125}I]glucagon (○) and yielded the presented stoichiometry. The arrow denotes the position of the DMPC alone, whether sonicated in the presence or absence of KBr.

centrifugation data. Figure 4 shows the plot of $s_{20,w}$ against solvent density. The intercept of this plot yields a value of $\bar{v}$ = 0.919 ± 0.005 mL/g. Although density-perturbation techniques rest on the assumption that there is no preferential interaction of the solute with solvent components (Cassassa & Eisenberg, 1964), the fit of the s values to a straight line as a function of ρ in Figure 3 is indicative that in this case the particle is not greatly affected by salt. In cases where there is a preferential solvation with one of the components, a marked curvature of these plots would result (see, for example, Aune and Timasheff, 1970). In the case of low-density lipoproteins, a system more analogous to ours, Nelson et al. (1974) found good agreement between the molecular weights derived by varying ρ with KBr in equilibrium studies and those obtained by other methods and also that the molecular weight was not affected by changes in salt concentration.

The estimates of $\bar{v}$ for the glucagon–DMPC complex of

TABLE I: Determination of the Molecular Weight of Glucagon–DMPC.

Technique(s) applied	Method of analysis	Mol wt of glucagon–DMPC complex
Equilibrium ultracentrifugation	Density perturbation with KBr	1.6 ± 0.2 × 10^6
Velocity ultracentrifugation	Density perturbation with KBr	1.37 ± 0.04 × 10^6
Sedimentation velocity and quasi-elastic light scattering	Svedberg equation	1.38 ± 0.08 × 10^6
Sedimentation velocity and viscosity	Scheraga and Mandelkern (1953), using β = 2.13 × 10^6	1.42 ± 0.10 × 10^6

0.919 ± 0.005 mL/g obtained by this density perturbation method are in good agreement with the estimates of 0.916 ± 0.003 mL/g from density gradient centrifugation as well as 0.925 ± 0.005 mL/g measured directly by pycnometry (see Methods).

Molecular Weight. Estimates of the molecular weight for the complex, determined by several methods, are summarized in Table I. For these calculations, $\bar{v}$ was taken as 0.919 mL/g. Except for the Scheraga–Mandelkern equation, the molecular weight calculated is the weight-average molecular weight. A value of $\beta = 2.13 \times 10^6$ was used in the Scheraga–Mandelkern equation, since it is known that the particle is an oblate ellipsoid with an axial ratio of close to 3 (Schachman 1959, p 239). The good agreement between the various methods suggests that the analysis is accurate and the lipoprotein preparation is homogeneous and unaffected by high concentrations of KBr. The equilibrium flotation molecular weight is in agreement with the estimates obtained using other techniques. The uncertainty in this estimate has been noted above.

Stoichiometry. Since high concentrations of KBr interfere with the Lowry protein method, and scattering complicates simple absorption measurements, more indirect methods of protein analysis were needed to determine the stoichiometry in the fractions from the density-gradient ultracentrifugation. We used both fluorescamine to detect the primary amino groups of glucagon and the tracing of glucagon with [^{125}I]-glucagon as detection methods. Although both have possible disadvantages in that (1) complex formation with lipid may shield some amino groups from the reagent or (2) iodinated glucagon may interact in a different manner from unmodified glucagon, the methods produced similar results with a stoichiometry of ~55/1 mol of lipid/mol of glucagon at the peak (Figure 4). Some distribution of stoichiometry is observed in this figure, although it should be noted that 70–80% of the complex was found in the fractions whose stoichiometry fell in the narrow range of 45–60 mol/mol. While density gradient equilibrium centrifugation will detect differences in the $\bar{v}$ arising from differences in composition it is not sensitive to the size of the particles, while gel filtration is sensitive to size but not composition. Figure 5 shows the elution pattern of complex applied to a column of Sepharose 4B. The phospholipid pattern shows one major peak, while the glucagon trace shows two components—complex and free glucagon. The stoichiometry across the peak shows a plateau at 55–60 mol/mol, although the leading edge of the peak shows an enhancement of lipid relative to protein. This may be due to the presence of particles

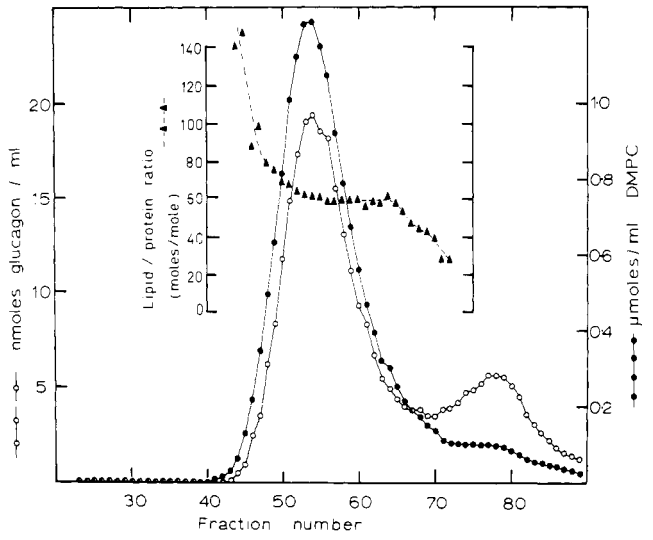

FIGURE 5: Sepharose 4B chromatography of glucagon lipoprotein. The 0.6 × 60 cm column was eluted with 0.1 M ammonium acetate (pH 7.4) at 4 °C at ~9 mL/h. Sixteen milligrams was applied and fractions of 0.75 mL were collected. Protein (○) was determined by the Lowry procedure using $NaDodSO_4$ as described under Methods. Phospholipid (●) was determined by the method of Bartlett (1959). The inner ordinates refer to the resulting stoichiometry (▲). The void volume of the column eluted in fraction 29 and the column volume in fractions 78–79.

of complex with a high molar ratio or may indicate the presence of some lipid vesicles eluting just before the complex.

Using the value of $\bar{v}_{DMPC} = 0.932$ and $\bar{v}_{glucagon} = 0.720$ (calculated from the amino acid composition) and the stoichiometry obtained above as 60 mol/mol, simple additivity of the $\bar{v}$ values on a weight basis yields a value of 0.916 for the complex, in good agreement with the measured values.

Stability. The flotation equilibrium experiments required run times in the order of days and it was necessary to ascertain that the complex is stable for such times. In previous experiments (Epand et al., 1977b), we had also required high concentrations of complex which were obtained by freeze-drying and reconstitution and, although the bulk physical properties remained unchanged, we wanted to check that this procedure had no effect on size and shape. To this end, we used autocorrelation spectroscopy (quasi-elastic light scattering) to monitor changes in the diffusion constant (Figure 2). We measured the diffusion constants of samples of complex at 20 and 14 °C, before and after freeze-drying and after 72 h at 6 °C (Table II). When the results were corrected for temperature and viscosity effects, there was no significant difference between 20 and 14 °C nor between fresh and freeze-dried samples. During storage in the cold a small amount of precipitate formed (probably glucagon, see above) which was removed by a low-speed spin before measurements were taken. A slight reduction (~10%) in the diffusion constant was observed. These results show that the complex remains largely unchanged over relatively long times and that lyophilization does not affect the model lipoprotein complex. As noted above, the complex is not significantly affected by these processes, although the glucagon in solution may be undergoing slow conformational and aggregation changes. The results also show that there are no gross differences between the particle size at 14 and 21 °C. This observation is of interest since there are changes in spectroscopic properties of the glucagon in the complex over this temperature range (Epand et al., 1977b). The value of $D_{20,w}$ used in molecular weight calculations was 2.00×10^{-7} cm^2/s, since free glucagon aggregates which were removed by centrifugation after "aging" (see Methods) could

TABLE II: Diffusion Coefficients of Model Lipoprotein Complex.[a]

State	$D_{20,w}$ ($\times 10^7$ cm^2 s^{-1})[b]	
	14 °C[c]	21 °C
Fresh	2.20 ± 0.03[d]	2.30 ± 0.05
Lyophilized	2.25 ± 0.02	2.16 ± 0.04
"Aged"[e]	2.00 ± 0.06	2.00 ± 0.04

[a] At ~3 mg/mL in 0.1 M ammonium acetate, pH 7.4. [b] Corrected for temperature and viscosity. [c] Temperature at which measurements were made. [d] Mean ± SEM, $n \geq 4$. [e] After 72 h at 0 °C a slight precipitate was removed by centrifugation; see text.

have contributed to the high values of D measured in the fresh solutions.

Dimensions of the Particle. Electron micrographs of negatively stained preparations of the complex (Figure 6a) reveal the presence of oblate ellipsoids lying both parallel to the plane of the grid (elliptical areas of low contrast) and perpendicular to the plane (apparently rod-like area of high contrast). Freeze-fracture electron micrographs (Figure 6b) reveal disk-shaped particles and depressions parallel to the fracture plane as well as views of particles whose major semiaxes are at different angles. No evidence of rods was seen in freeze-fracture micrographs, thus confirming the conclusions above concerning the interpretation of the apparently rod-shaped particles (in negatively stained preparations) as side-views of disks. Measurement of the particles in negatively stained micrographs yielded dimensions of 249 ± 6 × 68 ± 2Å (obtained exclusively from side views where the contrast was sufficient to delineate sharp edges). No attempt was made to quantitate the possible difference in the two major semiaxes. (The distribution of widths was essentially a bell-shaped curve, while for the length the curve was spread further, consistent with side views representing values between the two major semiaxes.) These dimensions yield a value of 3.64 as the axial ratio and a volume of 2.22×10^6 Å^3, of the particle seen in micrographs.

Hydrodynamic data yield estimates of these same parameters when a hydration value for the particle is assumed. Aune et al. (1977) found a value of 0.86 g of H_2O/g of DMPC, and this figure was used in the following treatment. The correspondence of the refractive index increment dn/dC of the complex (0.131 ± 0.003 mL/g) with that of DMPC alone (0.132 ± 0.002 mL/g, Kremer et al., 1977) together with the observations that all the head groups in the complex are exposed to the aqueous medium (Epand et al., 1977b) suggest that the hydration properties of DMPC in the complex will be similar to those for DMPC vesicles. The maximum error introduced by neglecting glucagon hydration will be ~5% [but, since glucagon is small (i.e., larger surface to volume ratio) compared to typical globular proteins (whose hydration is of the order of 0.2 g/g), its hydration will be closer to that of DMPC with a resulting decrease in the introduced error]; using the values $[\eta] = 6.24$ mL/g, $\bar{v} = 0.919$, and a hydration of 0.86 g/g, we can calculate Simha's constant to be 3.51 corresponding, in the case of an oblate ellipsoid, to an axial ratio of 3.13:1. From a knowledge of the mol wt and these same parameters, molecular dimensions may be obtained using the method described by Tanford (1961, p 356). A hydrated radius, R_0, of 99.1 Å is obtained, with a corresponding molecular volume of 4.08×10^6 Å^3. If the stain in the electron micrographs is able to penetrate the hydration shell, then the dimensions obtained from them will correspond to the unhydrated particle. The volume of the equivalent hydrated particle is obtained by multiplying by $\bar{v}_2 + \delta_1 v_1^0 \sqrt{v_2}$ (Tanford, 1961,

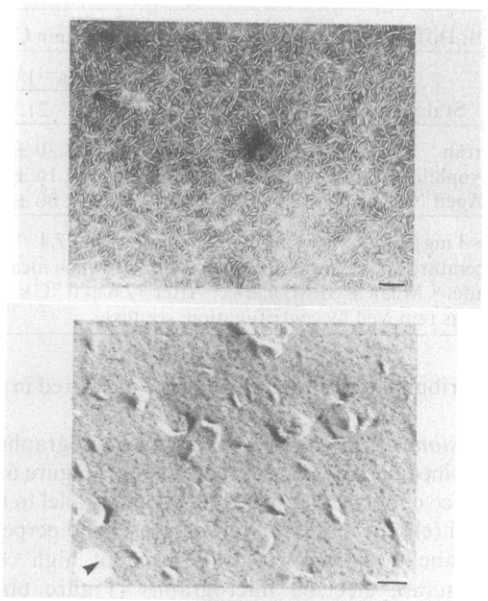

FIGURE 6: (a, Upper) Electron micrograph of glucagon lipoprotein negatively stained using 2% ammonium molybdate. The bar represents 1000 Å. (b, Lower) Electron micrograph of a freeze-fracture replica of the glucagon lipoprotein. The arrow indicates the angle of shadowing and the bar represents 500 Å.

eq 20-6) to yield 3.96×10^6 Å^3, in excellent agreement with the hydrodynamic data.

From $s^0_{20,w}$ and $D^0_{20,w}$, the ratio of frictional coefficients f/f_{min} can be calculated. This ratio is found to be 1.34 and 1.35, respectively, corresponding to an $f/f_0 = 1.08$ (using $\delta_1 = 0.86$) and an axial ratio of 2.65:1 for an oblate ellipsoid.

Table III summarizes the data for the complex and compares them with those for unilamellar phospholipid vesicles and apolipoprotein–DMPC complexes.

TABLE III: Comparison of the Physical Properties of Glucagon–DMPC with Those of Serum Apolipoprotein–DMPC and of Sonicated Lecithin Vesicles.

Property	Glucagon–DMPC	Sonicated lecithin vesicles	Serum apolipoprotein–DMPC
$[\eta]$ (dL/g)	0.062	0.0403[a]	
$D^0_{20,w}$ ($\times 10^7$ cm^2/s)	2.00	1.72[b]	5.24[d]
$s^0_{20,w}$	9.2 S	6.59 S[b,c]	5.06 S[b]
			3.84 S[d]
$\bar{v}$ (mL/g)	0.92	0.96[b,c]	0.92[b,d]
Mol wt	1.4×10^6	1.9×10^6[a]	4.4×10^5[b]
		2.7×10^6[b]	$\sim 2.4 \times 10^5$[d]
f/f_{min}	1.34	1.2[a]	
Axial ratio (oblate ellipsoid)	2.9:1	1	2:1[e]
Molar ratio of lipid to protein	57:1		52:1[b]
			100:1[d]
Dimensions	250 × 70 Å	105 Å, rad[a]	110 × 55 Å[e]
		125 Å, rad[b]	
Shape	Oblate ellipsoid	Sphere	Oblate ellipsoid

[a] Newman and Huang (1975) for egg-yolk lecithin. [b] Aune et al. (1977) for apolipoprotein C-III and DMPC. [c] Measured at 23 °C. [d] Hauser et al. (1974) for apoprotein from porcine high-density lipoprotein and DMPC. [e] Atkinson et al. (1976), same system as in footnote *d*.

Discussion

The relatively high axial ratio obtained from electron micrographs is due to the fact that the image is that of the unhydrated particle. The thickness of the hydration shell (predicted using 0.86 g/g as the hydration value) around the EM image is ~15 Å. This reduces the axial ratio to 2.92 comparable to the mean (2.89) value obtained from Simha's constant ($a/b = 3.13$) and f/f_0 ($a/b = 2.65$). The surface area of the unhydrated particle is 1.05×10^5 Å^2, and from the molecular weight and stoichiometry contains 1810 DMPC molecules. The surface area of DMPC in the gel state is 50 Å^2 (Curatolo et al., 1977). In the complex, the molecular properties of DMPC seem relatively unaltered, apart from a restriction of the lateral diffusion of the lipid molecules; the aromatic residues of glucagon are embedded in the hydrocarbon volume of the complex, although it is not possible to determine where on the surface they are located (Epand et al., 1977a,b). If the surface area of the DMPC molecule is unaltered in the complex, then the surface area of 1810 DMPC molecules is 9.05×10^4 Å^2. The contribution to the surface area by glucagon may be estimated from its dimensions assuming it to be largely α-helical (Epand et al., 1977a). A 29 amino acid length of helix approximates to a cylinder of $h = 43$ Å and $d/2 = 5.2$ Å. If the glucagon is embedded with the axis of the cylinder parallel to the plane of the lipid bilayer, then 32 molecules will contribute 1.43×10^4 Å^2. If this is added to the area occupied by the lipid, the total surface area will be 1.05×10^5 Å^2, the value obtained from the EM. The model proposed by Segrest (1977) for phospholipid–amphipathic helix association, the "bicycle tyre" model, locates the apolipoprotein at the rim of the oblate ellipsoid typical of such complexes. The particle formed by glucagon and DMPC is not only too thin to be a collapsed vesicle but also contains insufficient lipid for such a structure. The accessibility of all the lipid head groups to externally added paramagnetic ions shows that the lipid may be visualized as a single bilayer disk (Epand et al., 1977b). The exposure of the acylphospholipid chains around the edge of such a disk is energetically unfavorable and may be overcome by one or both of two mechanisms: interactions with the hydrophobic surface of an amphipathic helix or by drastically decreasing the radius of curvature at the rim. The surface area of the curved face of the complex (in this case seen as a cylinder with $d/h = 3.6$, volume $= 2.22 \times 10^6$ Å^3) is 3.26×10^4 Å^2. Much of this surface could be covered by the hydrophobic half of the glucagon helix which could occupy 2.25×10^4 Å^2 if all of the peptide was at the rim. The area occupied by the hydrophobic surface of glucagon could be increased somewhat by protruding side chains, and the area on the rim of the lipid bilayer may be decreased by a curvature of the phospholipid head group plane around the surface of (and possibly due to interaction of) protein and lipid, as suggested by Segrest (1977), who considered that the decreased radius of curvature would have the effect of concentrating the protein at the rim. The dimensions and composition of this model lipoprotein complex are consistent with Segrest's bicycle tyre model. In viewing this particle as a model lipoprotein, it is instructive to compare similar studies on the reconstitution of serum lipoproteins from their constituent apoproteins and lipids. In general, the nature of the particle produced in reconstitution studies depends on the initial ratio of the reactants. For example, the sedimentation coefficient of egg-yolk phosphatidylcholine vesicles increased linearly from 1.19 to 4.93 S as they were titrated with apo C-III and then remained constant even if the lipid protein ratio was increased (Morrisett et al., 1974). For the same apolipoprotein (apo C-III), however, the

pattern of interaction with DMPC is more complex. As the lipid was titrated with protein, the *s* value increased from 5.3 to 10 S until a critical lipid/protein ratio was achieved. Thereafter, further protein caused a decrease in *s* to 4.5 S. This was interpreted (Aune et al., 1977) as a breakdown of the DMPC vesicles to smaller particles. Since glucagon only interacts with phosphatidylcholines below their phase-transition temperatures (Epand et al., 1977a,b; Jones & Epand, 1977), egg-yolk PC could not be used (transition temperature ca. −7 to −15 °C) and DMPC was used. We have shown that lipid which is solubilized by glucagon is in the form of lipoprotein particles of uniform size, shape, and stoichiometry. However, in the case of an excess of lipid to glucagon above the stoichiometric ratio all the reactants are removed by centrifugation (Epand et al., 1977b), probably due to incorporation of the complex into the large multilamellar vesicles produced during vortexing (see Methods). The lipoprotein formed between DMPC and glucagon appears to be more homogeneous than that formed between DMPC and either the apoprotein from porcine high-density lipoprotein (Hauser et al., 1974) or apolipoprotein C-III (Aune et al., 1977). In the former case sedimentation equilibrium runs indicate size heterogeneity, while in the latter case this is indicated by the results from quasi-elastic light scattering. If, as in the case of the sequenced apolipoproteins (Shulman et al., 1974, 1975; Jackson et al., 1974; Brewer et al., 1974) only parts of the chain participate, then a number of lipid–protein configurations are possible (depending on which sections of helix are involved) giving rise to a heterogeneous particle population. The uniformity of the complex frormed by glucagon and DMPC may be the result of the participation of the whole peptide chain in amphipathic helix formation.

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