Biochimica et Biophysica Acta, 728 (1983) 293–304
Elsevier Biomedical Press

BBA 71566

INTERACTION OF α-LACTALBUMIN WITH DIMYRISTOYLPHOSPHATIDYLCHOLINE VESICLES

III. INFLUENCE OF THE TEMPERATURE AND OF THE LIPID-TO-PROTEIN MOLAR RATIO ON THE COMPLEX FORMATION *

IGNACE HANSSENS [a], WILLY HERREMAN [a], JEAN-CLAUDE VAN CEUNEBROECK [a], HUGO DANGREAU [a], CONSTANT GIELENS [b], GISELE PREAUX [b] and FRANS VAN CAUWELAERT [a]

[a] *Interdisciplinair Research Centrum, Katholieke Universiteit Leuven, Campus Kortrijk, B-8500 Kortrijk and* [b] *Laboratorium voor Biochemie, Katholieke Universiteit Leuven, Dekenstraat 6, B-3000 Leuven (Belgium)*

(Received July 30th, 1982)
(Revised manuscript received November 11th, 1982)

Key words: α-Lactalbumin; Dimyristoylphosphatidylcholine; Lipid-protein interactions; Vesicle breakdown

We investigated the interaction between α-lactalbumin and sonicated dimyristoylphosphatidylcholine at pH 4 and different temperatures. (1) At 23°C and lipid-to-protein molar ratios below 170, the interaction results in a disruption of the original vesicles to form smaller complex particles. By the sedimentation velocity method we determined for this particle a molar mass of $(1.05 \pm 0.16) \cdot 10^6$ g·mol^{-1}. The lipid-to-protein molar ratio within the complex particle is 70/1, as earlier estimated. It follows that there are approximately 1200 lipid and 17 α-lactalbumin molecules per particle. At molar ratios above 170, α-lactalbumin strongly associates with the vesicles. In this case the vesicle entity remains. The ability of α-lactalbumin to break up the vesicles at this temperature is determined by the number of protein molecules which are required in the complex particle. (2) By means of fluorescence polarization of the lipophilic probe 1,6-diphenyl-1,3,5-hexatriene and energy transfer of the tryptophan groups of the protein to 1,3-(1,1′-dipyrenyl)propane located in the hydrocarbon region of the vesicles, it is shown that with increasing temperature above 25°C, complexes of decreasing internal lipid-to-protein molar ratio are formed. However, by electron microscopy we show that the overall size of these complexes remains approximately the same, i.e., bars with dimensions 70 × 220 Å. A temperature-reversible transformation occurs between these complexes, which cannot be isolated by gel chromatography. In contrast, the complex of molar ratio 70/1 remains stable at lower temperatures.

Introduction

α-Lactalbumin is a coenzyme of galactosyltransferase in the lactose synthetase system [1]. At physiological pH, α-lactalbumin is known to be a peripheral, highly water-soluble protein. In an acidic medium, α-lactalbumin undergoes a conformational change: it does not denature at pH 4, but rather expands by about 20–30% [2]. We used α-lactalbumin and dimyristoylphosphatidylcholine as a prototype to study the influence of a protein conformational change, induced by pH, on the interaction between that protein and a phospholipid [3–5].

At physiological pH, α-lactalbumin adsorbs only to the outer surface of the phospholipid vesicles. At pH 4, it has an apolipoprotein-like behaviour: it interacts with the apolar phase of phosphatidylcholine vesicles to form particles which are

* Parts I and II of this series are Refs. 3 and 5, respectively.

significantly smaller than the original vesicles [3–5].

In interaction studies between apolipoproteins and phospholipids, it has been observed that the temperature and the lipid-to-protein molar ratio of the starting mixture determine the kind of complex formed. Apolipoprotein-AII associates with dimyristoylphosphatidylcholine liposomes to give complexes whose structure is determined by the temperature and ratio at which the reaction is conducted [6]. The isolated complexes which differ drastically in stoicheiometry and relative molecular mass remain unchanged even after 30 days at 4°C; also they resist a temperature scan up to 100°C [7]. Association of apolipoprotein A-I with dimyristoylphosphatidylcholine leads to the formation of isolatable complex particles which are unstable when heated to 70–90°C [8]. Dissociation of micellar complexes has been established just above the transition temperature of the choline with model proteins having an apolipoprotein-like behaviour [9–10].

Furthermore, with apolipoprotein A-I [11] and dimyristoylphosphatidylcholine vesicles there is a limiting lipid-to-protein molar ratio at which the vesicles are not reduced to so-called 'micelles' or 'micellar complexes' by the interacting apolipoprotein.

The present study is undertaken to explore the latter two aspects for the dimyristoylphosphatidylcholine-α-lactalbumin interaction at pH 4. Fluorescence polarization and energy transfer measurements above 25°C show that, at increasing temperature, complexes of decreasing molar ratio are formed. Gel chromatography and light scattering data demonstrate that at 23°C the small unilamellar vesicles break down only if by random association they can bind the number of α-lactalbumin molecules required in the complex particle. If a smaller number of protein molecules is associated with the vesicle, only certain membrane properties (e.g., permeability, intervesicular association) are changed. Comparing these data with similar data obtained with apoliprotein A-I [11], we establish that, upon interaction with the latter protein, the origin of the breakdown of dimyristoylphosphatidylcholine vesicles can be ascribed also to the stability of the resulting micellar complexes.

Materials and Methods

Materials

α-Lactalbumin from bovine milk and L-α-dimyristoylphosphatidylcholine were obtained from Sigma and used without further purification. 1,6-Diphenyl-1,3,5-hexatriene was purchased from Eastman Kodak Co. 1,3-(1,1′-Dipyrenyl)propane was synthesized as described before [12]. All protein solutions and lipid dispersions were 0.1 M in NaCl and 0.01 M in acetate buffer (pH 4).

Methods

Small unilamellar vesicles were prepared by sonication for 25 min above the transition temperature (T_t) with an ultrasonic desintegrator MSE 150 operated at maximum power. Large unilamellar vesicles were obtained by the method of Szoka and Papahadjopoulos [13]. Protein concentrations were determined by the fluorescamine method of Böhlen et al. [14]. Phospholipid analysis is based on the formation of phosphomolybdenum blue, using the procedure of Vaskovsky et al. [15].

The equipment for gel chromatography on Sepharose 6B was installed in a thermostatically controlled cupboard (2203 Minicoldlab, LKB). By this means, thermostatically controlled samples can be introduced onto the column and chromatographed without temperature fluctuations. Light scattering at 400 nm [3], steady-state fluorescence polarization of 1,6-diphenyl-1,3,5-hexatriene [5] and energy transfer between α-lactalbumin and 1,3-(1,1′-dipyrenyl)propane [12] have already been described in detail.

Analytical ultracentrifugation. Sedimentation velocities and diffusion coefficients [16] were measured using a Spinco Model E analytical ultracentrifuge (Beckman Instruments Inc.). The distances on the Schlieren patterns are measured using a Nikon profile projector Model 6C.

Sedimentation coefficients. Sedimentation runs were performed with a normal 12 mm cell at a rotor speed of about 59780 rev./min at 20°C. Sedimentation coefficients s are defined as $(dx/dt)/\omega^2 x$ and were calculated from linear plots of log x vs. time; x is the distance from the centre of rotation to the point of maximum ordinate of the Schlieren pattern. Values of s were extrapolated to give s° at zero concentration of the com-

plex using a linear least-squares fit. The extrapolated sedimentation coefficient was further corrected for the density and viscosity of the buffer solution used, by reducing to water according to the relationship:

$$s^\circ_{20,w} = s^\circ_{20}\frac{\eta_{20}(1-\bar{v}_{20}\rho_{20,w})}{\eta_{20,w}(1-\bar{v}_{20}\rho_{20})}$$

where η_{20} and ρ_{20} are respectively the viscosity and density of the suspending solution at 20°C; $\eta_{20,w}$ and $\rho_{20,w}$ are the corresponding values for water; $\bar{v}_{20}$ is the partial specific volume of the complex at 20°C.

Diffusion measurements. Diffusion studies were carried out under conditions similar to those for the sedimentation runs, except that a synthetic boundary cell and a rotor speed of 5227 rev./min were used. At this speed the concentration boundary did not move. Apparent diffusion coefficients were calculated by the maximum ordinate-area method, using the relationship

$$D = \frac{1}{4\pi t}\left(\frac{A}{H}\right)^2$$

where A is the area under the Schlieren peak and H is the height of the peak maximum. Apparent diffusion coefficients were extrapolated to infinite dilution to give D°_{20} values, which were reduced to the value in water, according to the relationship

$$D^\circ_{20,w} = D^\circ_{20}\frac{\eta_{20}}{\eta_{20,w}}$$

Density measurements and partial specific volume determination. The densities of the acetate buffer, ρ_{20}, and complex solutions, ρ, were determined using a digital density-measuring instrument DMA 02 C from Anton Paar K.G. Densities can be obtained with an accuracy up to 10^{-5} g/ml. The partial specific volume of the complex, $\bar{v}$, which is the increase in volume when 1 g of the complex is added to an infinitely larger volume of solvent is determined from $\rho = \rho_{20} + (1 - \bar{v}\rho_{20})c$. $\bar{v}$ is calculated from the plot of the density ρ versus concentration c.

The viscosity measurements. The viscosity of the suspending buffer (for s°_{20} and D°_{20} corrections) was determined using an Ostwald viscosimeter.

Results

Determination of the limit lipid-to-protein molar ratio to break up small vesicles at 23°C

Gel chromatography

The profile of the lipid and protein elution (Fig. 1) of dimyristoylphosphatidylcholine vesicles incubated with α-lactalbumin varies as a function of the starting molar ratio.

At a molar ratio of 50, no free lipid is observed.

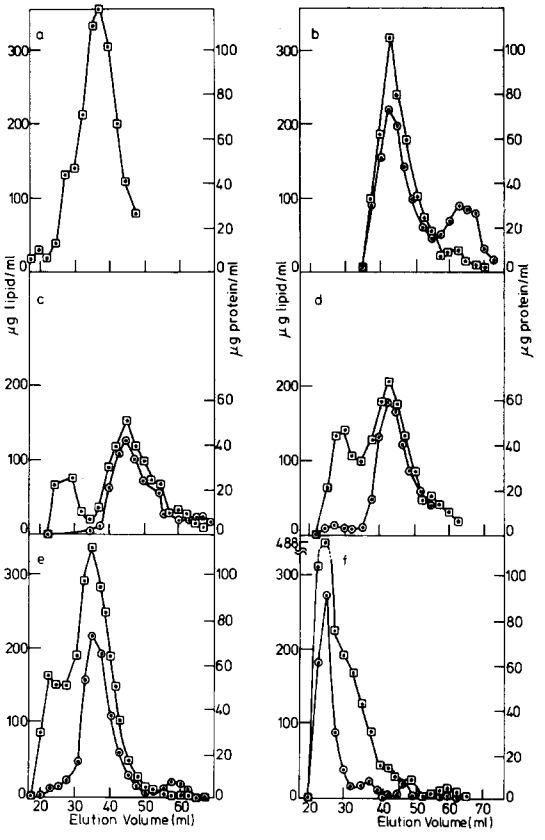

Fig. 1. Elution profiles of dimyristoylphosphatidylcholine (⊡) and α-lactalbumin (⊙) on a Sepharose 6B column of 1.6 cm diameter and 31 cm height. To 2 ml dispersion of small vesicles at pH 4 (0.01 M acetate buffer in 0.1 M NaCl) is added 0.2 ml of an α-lactalbumin solution. The mixture is incubated for 2 h before chromatographing at the incubation temperature. The lipid-to-protein molar ratios, N, final concentrations and incubation temperatures are: (a) $N = \infty$, 3 mg phospholipid/ml, 23°C; (b) $N = 50$, 1.5 mg phospholipid/ml and 0.6 mg α-lactalbumin/ml, 23°C; (c) $N = 90$, 1.2 and 0.28, 23°C; (d) $N = 150$, 2.0 and 0.28, 23°C; (e) $N = 200$, 3.0 and 0.3, 23°C; (f) $N = 200$, 3.0 and 0.3, 24.5°C. $V_0 = 22$ ml and $V_t = 62.5$ ml.

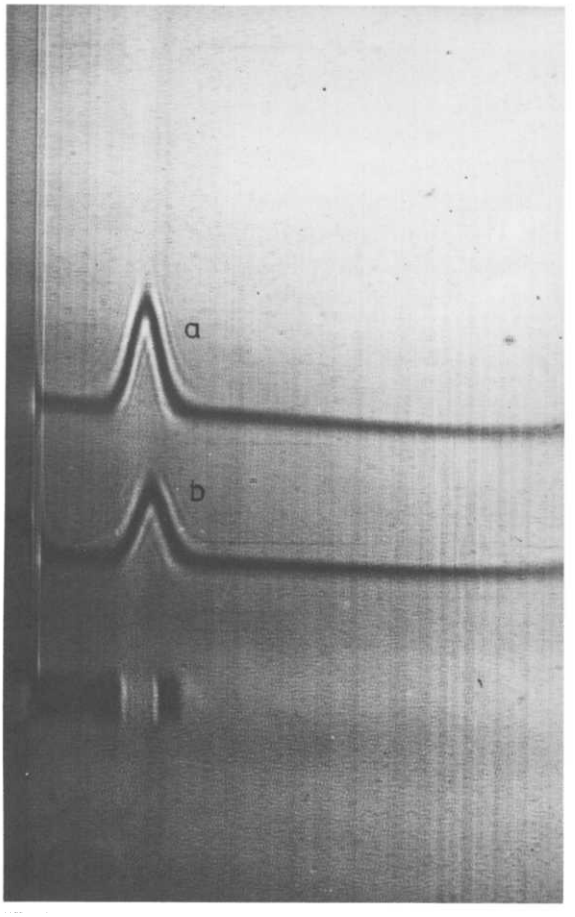

Fig. 2. Sedimentation pattern of the fraction of dimyristoylphosphatidylcholine-α-lactalbumin mixture which elutes between 37.5 ml and 50 ml on the Sepharose 6B column. Centrifugation time: 28 min, centrifugation speed: 59780 rev./min. Concentration: (a) 1.92 mg complex/ml; (b) 1.28 mg complex/ml; Temperature 20°C. Sedimentation direction from left to right.

The phospholipid elutes together with a part of the protein around 42.5 ml. These peaks are followed by the residual α-lactalbumin. A sedimentation pattern (Fig. 2) of the fraction between 37.5 ml and 50 ml shows that homogeneous particles are obtained. From the elution volume it is obvious that these particles are smaller than the original vesicles. At the molar ratios of 90 and 150, most of the protein molecules elute with the phospholipid in the complex fraction between 37.5 ml and 50 ml. However, in contrast to the situation of the molar ratio of 50, a residual amount of pure lipid migrates before this complex fraction at an elution volume of 30, lower than the elution volume of small vesicles, which has a maximum around 38 ml. Therefore, this pure lipid is present as larger structures. In the pattern, no residue of pure α-lactalbumin is observed. At the molar ratios 200 and above, α-lactalbumin elutes with dimyristoylphosphatidylcholine at the elution volume of the small unilamellar vesicles. As a consequence, the typical complex with lipid-to-protein ratio 70/1, is not observed. This result indicates that at 23°C and at these high molar ratios the vesicles are not broken up to small complex particles within the 2–3 h incubation period.

At 24.5°C and molar ratios up to 100, the gel chromatograms are identical to those at 23°C. However, at 24.5°C and a molar ratio of 200, the lipid and protein elute at the void volume of the column. As the pure vesicles do not aggregate to a great extent in the same circumstances, this coagulation is induced by the adsorbed protein.

In the concentration range of our experiments (1–3 mg lipid/ml), the phenomena at 23 and 24.5°C are partly reversible. A dimyristoylphosphatidylcholine-α-lactalbumin suspension of molar ratio 200 which after 2 h incubation at 24.5°C is brought to and chromatographed at 23°C, elutes as when mixed and incubated at the latter temperature. In reverse, even when the mixing and first incubation occur at 23°C, chromatographing at 24.5°C determines the elution pattern. Gel chromatograms of mixtures of large unilamellar vesicles and α-lactalbumin, incubated at starting molar ratios 300 and 500, were also obtained at 23°C. In both cases, the protein eluted with some lipid at about 42.5 ml. The elution volume and the internal lipid-to-protein molar ratio 70/1 correspond to the typical complex.

Light scattering

The light scattering of a vesicle suspension has been followed as a function of time after the injection of α-lactalbumin solution (Fig. 3). The lipid-to-protein molar ratios are varied by changing the α-lactalbumin concentration using a constant amount of sonicated vesicles. At 23°C, the injection of α-lactalbumin immediately causes an increase in the light scattering. With the smaller molar ratios (under 200), the light scattering subse-

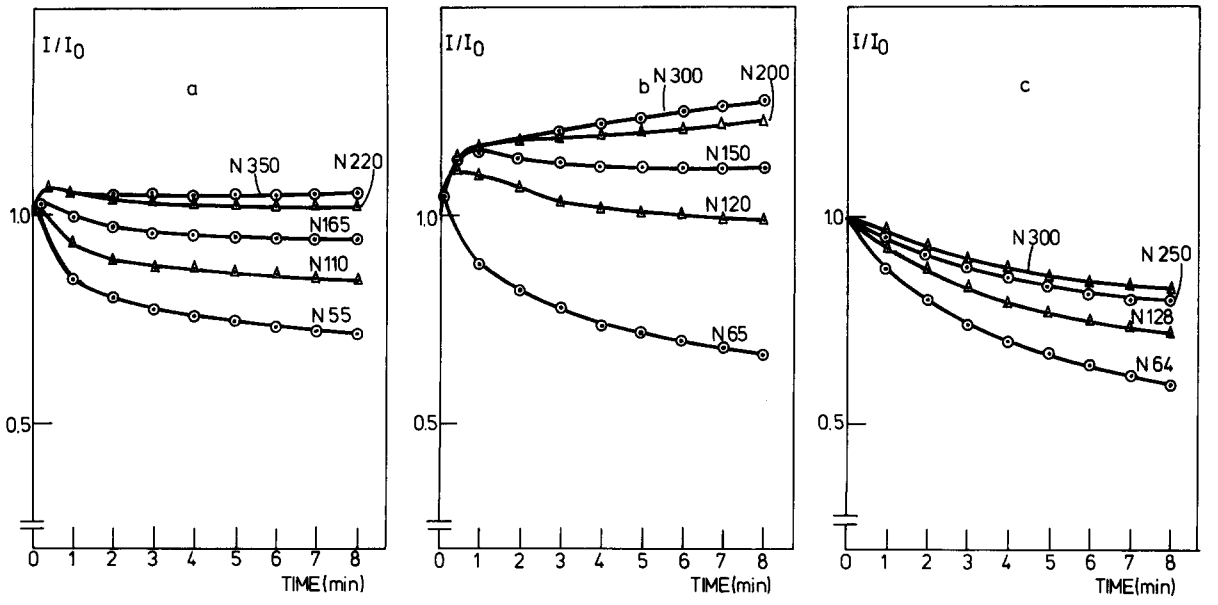

Fig. 3. Light scattering at 400 nm of vesicle suspensions after the injection of an α-lactalbumin solution. At time zero, 0.2 ml of an α-lactalbumin solution is added to 2 ml vesicle suspension (pH 4) of 2.15 mg dimyristoylphosphatidylcholine/ml. N is the lipid-to-protein molar ratio of the mixture. Experiments a and b are with sonicated small unilamellar vesicles respectively at 23°C and 24.5°C; for the experiment c large unilamellar vesicles are used at 23°C. I is the intensity of the scattered light. I_0 is the scattering of 2 ml vesicle suspension diluted with 0.2 ml water.

quently decreases to values which are lower than for the dispersions of pure vesicles. The results agree with the gel chromatographic data on the formation of small complex particles.

With the larger molar ratios (over 200), the intensity of the scattered light does not return below the value of pure vesicles; a quasi-constant light scattering, which is about 5% higher than for the vesicle suspension without α-lactalbumin, is obtained within 10 min after mixing. The rather small increase can be explained by simple adsorption of α-lactalbumin to the vesicles [17]. The adsorption of the protein to the vesicles appears as a process which preceeds the vesicle disruption, and Fig. 3 indicates that at higher lipid-to-protein molar ratios, the adsorbed α-lactalbumin does not readily disrupt the vesicles.

At 24.5°C the increase in light scattering upon injection of α-lactalbumin is more pronounced than it is at 23°C. At molar ratios above 150, a prolonged increase of the intensity of light scattering is observed and the suspension becomes turbid. However, such turbid suspension clears up rapidly on cooling to 23°C.

On adding α-lactalbumin to large unilamellar vesicles at 23°C an immediate decrease of the light scattering occurs. It is important to note that the light scattering also decreases at molar ratio 300, at which the protein is not able to break up small vesicles. Also, the lipid-protein complexes formed with the large unilamellar vesicles are identical to those in the case of small vesicles. These data agree, therefore, with the gel chromatographic data above.

Mass of the complex particle isolated at 23°C

By chromatography on Sepharose 6B, the complex particles fraction eluting between 37.5 ml and 50 ml has been isolated from a concentrated solution containing 12 mg/ml lipid and 3 mg/ml α-lactalbumin. The Schlieren sedimentation patterns of this large fraction are found to be reasonably symmetrical (Fig. 2), indicating that the isolated complex particles are homogeneous. The weight-

average particle molar mass is calculated from the determined values of the sedimentation coefficient $s^{\circ}_{20,w} = 8.52 \pm 0.06$ S; the diffusion coefficients $D^{\circ}_{20,w} = (2.19 \pm 0.08)\ 10^{-7}$ cm^2/s; the partial specific volume of the complex $\bar{v} = 0.91 \pm 0.01$ cm^3/g and the density of water $\rho_{20,w} = 0.99823$ g/ml. These parameters combined in the Svedberg equation:

$$M = RTs^{\circ}_{20,w}/D^{\circ}_{20,w}(1 - \bar{v}_{20}\rho_{20,w})$$

yield a particle molar mass of $(1.05 \pm 0.16) \cdot 10^6$ g/mol. From this mass and the lipid-to-protein molar ratio within this complex of 70/1, it can be estimated that such an entity consists of about 1200 phospholipid and 17 α-lactalbumin molecules.

Watts et al. [18] determined that sonicated dimyristoylphosphatidylcholine vesicles have 2850 ± 140 lipid molecules per vesicle. Since our preparation procedure is very similar to theirs and since from electron microscopy we determined 280 ± 30 Å as the diameter of the vesicles, while they obtained a mean value of 300 Å, we adopted the above number to calculate that the lipid content in the complex particle is only 42% of that in a vesicle. Therefore, after disruption of a vesicle by 17 α-lactalbumin molecules, large fragments of vesicles remain and can recombine to form larger aggregates or liposomes.

The volume of one micellar or complex particle can be calculated from the sedimentation analysis and the gel chromatographic data. From the partial specific volume of the complex, $\bar{v}_{20} = 0.91$ cm^3/g, one calculates that $\rho = 1.099$ g/cm^3. The volume of 1 mol, V_m, is $M/\rho = 9.55 \cdot 10^5$ cm^3/mol, or for one particle $V = 1.6 \cdot 10^{-18}$ cm^3. After calibrating the elution pattern of the Sepharose column with some reference proteins, a Stokes' radius of 68 Å has been deduced for the complex particle. This yields a volume, V, of $1.32 \cdot 10^{-18}$ cm^3, in good agreement with the value obtained from the sedimentation analysis.

The mass of the complex particles at 27, 30 and 33°C is not determined. However, the electron microscopic graphs of mixtures incubated at 23°C with a starting molar ratio of 15 and brought to 27, 30 and 33°C for 1 h show similar bar-shaped entities as at 23°C. We have previously shown [3] by electron microscopy that the complex particles at 23°C had dimensions of 70 × 220 Å. The mean dimensions of the particles at 27, 30 and 33°C are, respectively, 70 × 200 Å, 70 × 300 Å, 70 × 250 Å. This indicates that the size of the complex particles does not change drastically. On the other hand, as will be seen further, the lipid-to-protein molar ratio within the complex decreases strongly.

Influence of the temperature on the composition of the complex

Gel chromatography

At temperatures below 23°C. In these experiments small unilamellar vesicles (1.7 mg/ml) and α-lactalbumin (0.7 mg/ml) were mixed, incubated for 2 h and chromatographed at 20, 16 and 12°C. At 20 and 16°C, the lipid elutes with part of the α-lactalbumin at the elution volume of the complex. In the complex fraction, the lipid-to-protein molar ratio remains 70/1, as is found at 23°C. On mixing and incubating at 12°C, the fraction of lipid and protein eluting in a common peak is strongly reduced. If a first incubation at 23°C is followed by a second one at 12°C, the chromatogram obtained at the latter temperature does not differ from that at 23°C: it indicates that the complex particles resist the cooling process.

At temperatures above 23°C. The lipid and protein analysis of the eluents after incubation and chromatographing a mixture of molar ratio 15 at 23°C and above that temperature is presented in Fig. 4. In these experiments, low concentrations of lipid and protein are used to prevent precipitation. As a consequence, larger volumes of the dispersions are chromatographed to permit the product analysis.

The analysis demonstrates that the composition of the complex changes as a function of the temperature. At 23°C, apart from the peak for free α-lactalbumin, a common maximum is found for the lipid and protein, as has been described before. After the incubation and chromatography at 28°C, the lipid and bound α-lactalbumin peak move to lower elution volumes; also, the maximum of the lipid no longer coincides with the maximum of the protein intermediate peak and the fraction of α-lactalbumin in the intermediate peak is lower than at 23°C. In an analogous experiment carried out at

33°C, no common peak is found for the two substances. A lipid maximum is found at the void volume and the lipid peak tails over the chromatogram. Besides the high protein concentration which elutes at the total column volume, a low protein concentration is found over the whole chromatogram.

Although no further product analysis has been carried out, the elution profiles showing the light transmission at 254 nm indicate that above 23°C the temperature effect is reversible. A dispersion of lipid and α-lactalbumin, which after a 2 h incubation period at 33°C is brought to 23°C for 15 min and then chromatographed, shows the same light transmission profile as a dispersion which has been incubated immediately at 23°C. The effect in increasing the temperature after an incubation at 23°C shows the same reversibility.

Fluorescence polarization

In Fig. 5 fluorescence polarization data of diphenylhexatriene are shown as a function of the starting molar ratio, at different temperatures. The measurements were carried out after an incubation period of 20 h, to be certain that the interactions reached equilibrium. It is observed that at 25°C no change in polarization is observed for molar ratios up to 70, indicating that all the phospholipids are in an identical environment, namely the complex. With an increasing excess of lipid (molar ratio over 70) the polarization decreases caused by the presence of an increasing fraction of residual non-complexed lipid. These results agree with the gel chromatographic data.

From the fluorescence polarization curve it is established that at 29, 30 and 33°C, complexes of a molar ratio 30, 25 and 15 are formed. No fluorescence polarization data are available below 23°C, as the difference between the polarization in the vesicles and in the complex becomes too small at that temperature.

Energy transfer from α-lactalbumin to the 1,3-(1,1'-dipyrenyl)propane probe

The transfer of excitation energy from α-lactalbumin tryptophan residues (excitation 292

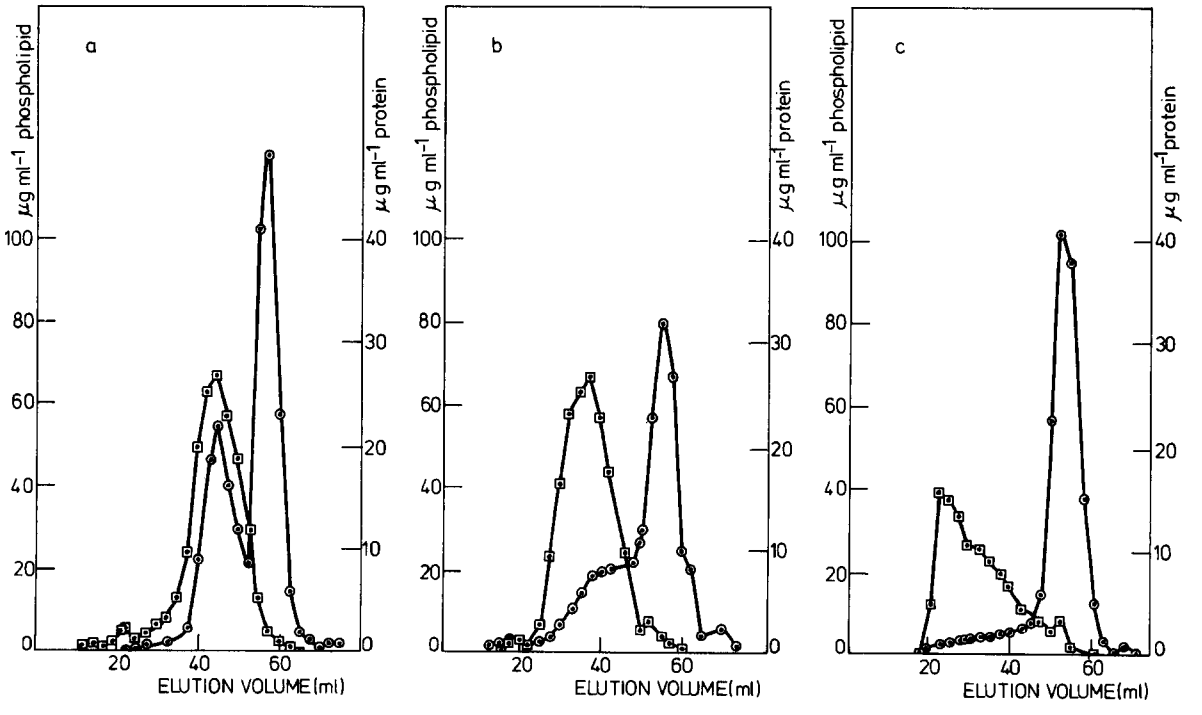

Fig. 4. Elution profiles of dimyristoylphosphatidylcholine (⊡) and α-lactalbumin (⊙) at pH 4 on a Sepharose 6B column. The mixtures contain 0.28 mg lipid/mg and 0.40 mg protein/ml, which corresponds to a molar ratio 15. The incubation and chromatographing temperatures are respectively (a) 23, (b) 28 and (c) 33°C.

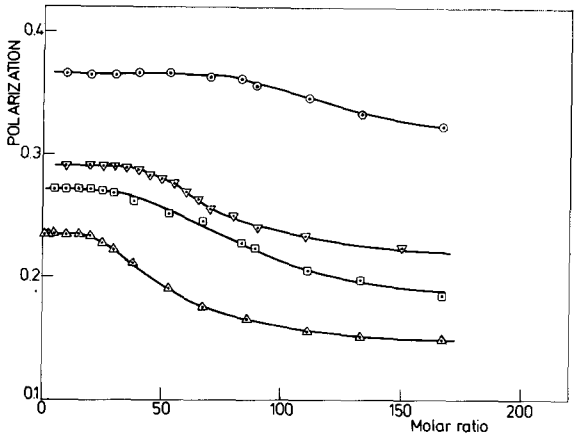

Fig. 5. Fluorescence polarization, P, of 1,6-diphenyl-1,3,5-hexatriene as a function of the lipid-to-protein molar ratio N. The lipid concentration is always 0.1 mg/ml; the α-lactalbumin concentration varies. Before measuring, the mixtures are incubated during 20 h at the desired temperature: (⊙) 25, (▽) 29, (⊡) 30 and (△) 33°C.

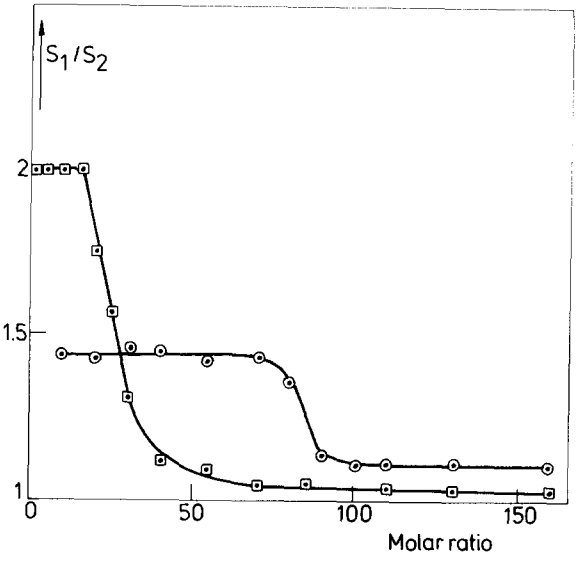

Fig. 6. Energy transfer from α-lactalbumin to 1,3-(1,1′-dipyrenyl)propane located in the apolar phospholipid region as a function of the lipid-to-protein molar ratio N, after 20 h incubation at the desired temperature: (⊙) 25°C and (⊡) 33°C. The lipid concentration is always 0.15 mg/ml; the α-lactalbumin concentration varies. S_1/S_2 represents the area under the emission spectrum of dipyrenylpropane in presence of α-lactalbumin to that measured in absence of the protein.

nm) to lipid inserted 1,3-(1,1′-dipyrenyl)propane was demonstrated [12]. α-Lactalbumin only adsorbs to the surface of the vesicles at pH 7: under this condition no energy transfer is measured because the distance between the probe in the lipid bilayer and the protein is too high. However, at pH 4 the protein penetrates into the bilayer and the distance between the probe molecules and the protein is decreased so drastically that a strong energy transfer is measured. We used this property to measure the molar ratio within the lipid-protein complex. When increasing amounts of protein are added to the same quantity of lipid, more and more protein becomes incorporated and the amount of energy transfer increases. However, at molar ratios lower than the lipid-to-protein molar ratio of the complex, the excess protein is not incorporated in the apolar phase and a level of constant high energy transfer is observed.

The results of the energy transfer at 25 and 33°C as a function of the molar ratio are depicted in Fig. 6. S_1/S_2 represents the ratio of area under the emission spectrum of dipyrenylpropane in presence of α-lactalbumin to that measured in absence of the protein. An area ratio equal to 1 indicates that no energy transfer occurs. From the figure it is ascertained that, at 25°C, a constant amount of energy is transferred from α-lactalbumin to the embedded probe up to a molar ratio of 70. As a consequence, the results confirm that at lipid-to-protein molar ratios below 70, the excess α-lactalbumin does not interact with the lipid phase. At 33°C, the formation of complexes of smaller molar ratio is also confirmed.

Light scattering on temperature change

The temperature-induced conversion of this complex can also be followed by light scattering.

In a first experiment (Fig. 7), different amounts of α-lactalbumin are added to a suspension of small vesicles. After a 2 h incubation period at 23°C to allow complex formation, the mixture is brought to 20°C and the scattering is measured during the subsequent temperature scan. At higher molar ratios the scattering starts to increase at 25.5°C. The increase is relatively more important at the higher molar ratios, and is not observed at the lower molar ratios. In view of the fluorescence polarization data, which demonstrate that at higher temperature complexes of smaller molar ratio are formed, the increase in light scattering of the lipid-rich solutions should be attributed to the

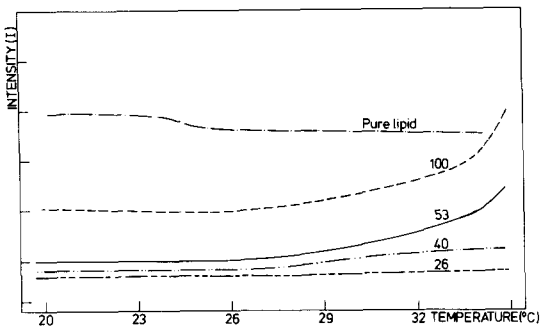

Fig. 7. Temperature scan of light scattering at 400 nm of sonicated dimyristoylphosphatidylcholine vesicles and its mixtures with α-lactalbumin. Scan rate, ±1 K/min. Angle of scattering, 90°. Previously the mixtures are incubated at 23°C to allow complex formation. The final lipid concentration is 1.0 mg/ml, the protein concentration varies to obtain the molar ratios (----) 26, (-·-·-) 40, (———) 53, (------) 100 or (-·-) pure lipid. All scans are upwards.

reformation of vesicular lipid particles. In presence of excess α-lactalbumin, the lack of changes in light scattering indicates that lipid lost from the complexes immediately binds to the excess protein.

To obtain some indication on the rate of the conversion, the scattering is followed as a function of time at different temperatures (Fig. 8). At the start of the reaction, 0.5 ml lipid-protein complex

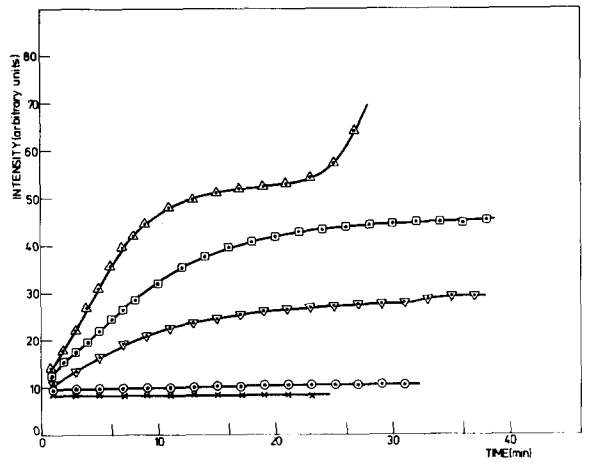

Fig. 8. Scattering change at 400 nm on transfering the dimyristoylphosphatidylcholine complex (0.5 ml buffer solution containing 3.0 mg lipid and 0.9 mg protein) incubated at 23°C to a buffer solution (2 ml) at higher temperature. The resulting temperature is, respectively, (×) 23°C, (⊙) 25°C, (▼) 28°C, (⊡) 30°C and (▲) 33°C.

(molar ratio 70) which has been maintained at 23°C is introduced into 2 ml buffer solution at higher temperature. The temperature after mixing is noted.

In these experiments, again, the light scattering does not change below 25°C, indicating that below that temperature the complex micelles are stable. At higher temperature an immediate increase in turbidity starts. The lipid turbidity, which is obtained after 15–20 min, increases the higher temperature. These data correspond to an increasing amount of vesicular lipid particles that can be formed when complexes of higher molar ratio transform to compexes of lower molar ratio. By electron microscopy of negatively stained samples, it has been observed that in the conditions of the latter light scattering experiment at 30°C, liposomes of 100–200 nm in diameter with a limited number (2–4) of bilayers are formed.

Discussion

α-Lactalbumin at pH 4 has, in many respects, a behaviour that is very similar to that of apolipoprotein A-I and glucagon at pH 7: they disrupt the dimyristoylphosphatidylcholine vesicles at 24°C to form smaller lipid-protein 'micellar' complexes of definite stoichiometry, size and shape [3,11,19]. These properties are compared in Table I. These micellar complexes are not only found by break up of small vesicles, but also of large unilamellar and multilamellar vesicles. We have found by gel chromatography, electron microscopy and by fluorescence polarization that the micellar complexes found from small and large vesicles have identical properties [20].

Using egg yolk phosphatidylcholine vesicles, Hoff et al. [21] observed a progressive flattening of the small vesicles with increasing apolipoprotein C-III concentration. Therefore, Segrest [22] correlated the micellar complex formation with the destabilization of the vesicular complex: incorporation of the protein molecules in the outer leaflet of the bilayer expands it and induces a great stress. If too many protein molecules are incorporated, a disruption of the initial vesicle structure results in formation of small micellar complexes.

In the interaction studies with small dimyristoylphosphatidylcholine vesicles and apoli-

TABLE I

PROPERTIES OF MICELLAR COMPLEXES OF α-LACTALBUMIN, APOLIPOPROTEIN A-I (APO A-I) AND GLUCAGON WITH DIMYRISTOYLPHOSPHATIDYLCHOLINE AT 24°C

Property	α-Lactalbumin [a] complex pH 4	Apo A-I [b] complex pH 7	Glucagon [c] complex pH 7
Particle molar mass (10^6 g/mol)	1.05	0.26	1.4
Lipid per particle	1200	280	1870
Protein per particle	17	3	34
Lipid/protein in particle	70/1	95/1	55/1
Electron micrograph dimension (Å)	70×220	50×150	70×250
Electron micrograph shape	disc or rod	disc ($a/b = 3/1$)	disc
Break-up molar ratio	170	not sharp	

[a] Data from Ref. 3 and this paper.
[b] Data from Ref. 11.
[c] Data from Ref. 19.

poprotein C-III, a marked reduction of the vesicle size starts at a much lower amount of the protein than with the egg yolk phosphatidylcholine [23]. Aune et al. [23] suggested that rigid, saturated phosphatidylcholines cannot accumulate as much proteins as can loosely packed unsaturated lipids.

For apolipoprotein A-I and dimyristoylphosphatidylcholine, Jonas et al. [11,24] showed that the formation of micellar complexes occurred preferentially for larger vesicles and small starting lipid-to-protein molar ratio. At high starting ratios and small vesicle radius, preferentially vesicular complexes are formed. Following the viewpoint of Segrest [22] and Aune [23], they concluded that the critical factor which determines the formation of either vesicular or micellar complexes is the surface concentration of apolipoprotein A-I on the vesicles. Therefore, under identical conditions, larger vesicles or liposomes with a smaller total surface will give more micellar complexes. We believe that the results of our paper allow us to go one step further: namely, that for saturated phosphatidylcholine vesicles the surface concentration of the protein, at which vesicular complexes are broken down to micellar complexes, is determined by the number of protein molecules that is present in the micellar complex particle. In this study, the micellar complex contains 17 α-lactalbumin molecules and about 1200 lipids molecules. If, therefore, on adding α-lactalbumin to dimyristoylphosphatidylcholine 17 α-lactalbumin molecules can adsorb randomly per vesicle, disruption of the vesicle will occur and the micellar complex originates by intravesicular rearrangment. If less than 17 molecules are adsorbed, the vesicle resists disruption. The proposition stated above is derived from the close relationship that is found experimentally between the number of protein molecules in the micellar complex and the number of protein molecules needed to destabilize the vesicles. Indeed, as a sonicated vesicle comprises 2850 lipid molecules, 17 protein molecules will randomly adsorb per vesicle at a starting molar ratio 170. Experimentally, we found formation of micellar complexes below molar ratios 170–200, while above 200 the protein eluted with the small vesicles at the elution volume of the original vesicles (vesicular complexes). We also found, in agreement with Jonas et al. [24], that in a suspension of large unilamellar vesicles, small complexes were formed with α-lactalbumin even at a molar ratio 500. In general, we suggest that near the transition temperature the phosphatidylcholine molecules already have a higher free energy in the vesicle than in the micellar complex and, therefore, the vesicles do not need an extra destabilization (as proposed by Segrest [22]) in order to break down to complex particles. Two experimental results are in favour of this: first, the constant appearance of the complex particles, independent of the starting vesicle type [20] or lipid-to-protein molar ratio below 170. Secondly, large unilamellar vesicles, in which the lipid has a lower enthalpy state [20] and probably a lower free energy than in small vesicles [25], still form micellar complexes at very high molar ratios.

The data of Jonas et al. [11,26] on the apolipoprotein A-I-dimyristoylphosphatidylcholine micellar complex are not in contradiction with our

proposal. These authors found three protein molecules per complex particle. On the other hand, they calculated for mixtures of different molar ratio that the probability of finding three or less apolipoprotein A-I molecules per vesicle corresponds to the experimentally observed percentages of apolipoprotein A-I in vesicular complexes. The disruption of the vesicles occurs, as suggested by Segrest, in two steps: adsorption of the protein to the vesicles (a fast step, observed by us in batch calorimetric experiments [20]), and a slower intravesicular rearrangement. Two observations must be made concerning this rearrangement.

(1) A spontaneous intermembrane transfer of some membrane proteins [27–28] and free exchange of apoproteins among lipoproteins and vesicles [29] has been demonstrated. If free transfer of α-lactalbumin results in an accumulation of the protein on some vesicles, an ultimate breakdown of those should occur even at a lipid-to-protein molar ratio above 200. Since no small particles are chromatographed at the molar ratio 200, it means that the protein does not accumulate on some vesicles. Even at 24.5°C, the temperature at which an α-lactalbumin-induced coagulation was observed, such protein accumulation, creating favourable conditions for complex formation, does not occur within a 2 h incubation period.

(2) At dimyristoylphosphatidylcholine-α-lactalbumin molar ratios between 70, the molar ratio in the complex particle, and 170, the molar ratio of the stable vesicular complex, there is an excess of lipid after the formation of lipid-protein particles. In the gel chromatography of these suspensions an amount of protein-free lipid elutes with the void volume of the column. It indicates that the excess lipid is no longer present as small vesicles. The creation of the complexes is therefore accompanied by an agglomeration of the excess lipid of different vesicles. If, however, the adsorbed protein redistributes freely among vesicles in order to agglomerate into complex particles, it should be expected that an important fraction of the small vesicles remains unchanged after complexation.

By several techniques we established that different complexes are formed at high temperature. These complexes are able to convert one to the other. From fluorescence polarization it was derived that the complexes have lipid-to-protein molar ratios 70, 30, 25 and 15 at, respectively, 25, 29, 30 and 33°C. The results at 25 and 33°C were confirmed by energy transfer from α-lactalbumin to a probe incorporated in the lipid phase. From these data it is expected that, upon chromatographing a dimyristoylphosphatidylcholine-α-lactalbumin mixture of molar ratio 15, the lipid and the protein should coincide at 33°C, while at 28°C a larger α-lactalbumin fraction is expected to elute with the lipid than at room temperature. In practice, at 33°C the lipid and protein are completely separated, while at 28°C the maximum of the lipid does not coincide with the maximum of the associated protein and the lipid-associated protein fraction is also lower than expected. An explanation for this contradictory result can be found in the instability of the complex particles. The 15/1-complex at 33°C is not stable enough to allow its separation by gel chromatography, and by poor stability of the complex at 28°C the phospholipid and associated α-lactalbumin are partly separated. As a consequence, not only are complexes of smaller molar ratio formed at higher temperature, but the complexes themselves become unstable. Although the internal lipid-to-protein ratio in the micellar complex decreases drastically with temperature, the overall dimensions of these complexes do not change drastically as measured by electron microscopy. The consequence of this is that the number of α-lactalbumin molecules per complex particle increases markedly. These complex particles all resemble bars with small variations in length but always with a width of approx. 70 Å, which was also found for the glucagon micellar complex (Table I). At the moment, our data do not allow discrimination between a rod-like or discoidial shape for these bars.

From these observations it is quite obvious that it is difficult to interpret scans of differential calorimetry (or scans of other physical properties as a function of temperature) of lipid-protein complexes.

In the last decade, many studies have been carried out on delivery of drugs entrapped in liposomes. It is observed that after an intravenous injection, large liposomes clear faster from the blood than small vesicles. Scherphof [30] also observed a massive transfer of the liposomal lecithin

to plasma high-density proteins. Our present study contributes to an understanding of these phenomena.

Acknowledgements

This study was supported by a grant of the 'Nationaal Fonds voor Wetenschappelijk Onderzoek'. We are grateful to Mrs. G. Dewitte for excellent technical assistance.

References

1 Brew, K., Vanaman, T.C. and Hill, R.L. (1968) Biochemistry 59, 491–497
2 Kuwajima, K., Nitta, K. and Sugai, S. (1975) J. Biochem. 78, 205–211
3 Hanssens, I., Houthuys, C., Herreman, W. and Van Cauwelaert, F.H. (1980) Biochim. Biophys. Acta 602, 539–557
4 Herreman, W., Van Cauwelaert, F.H. and Hanssens, I. (1981) Biochem. Intern. 2, 237–242
5 Herreman, W., Van Tornout, P., Van Cauwelaert, F.H. and Hanssens, I. (1981) Biochim. Biophys. Acta 640, 419–429
6 Massey, J.B., Rohde, M.F., Van Wimble, W.B., Gotto, A.M., Jr. and Pownall, H.J. (1981) Biochemistry 20, 1569–1574
7 Massey, J.B., Gotto, A.M., Jr. and Pownall, H.J. (1981) Biochemistry 20, 1575 ≠ 1584
8 Tall, A.R., Small, D.M., Deckelbaum, R.J. and Shipley, G.G. (1977) J. Biol. Chem. 252, 4701–4711
9 Epand, R.M., Jones, A.J.S. and Schreier, S. (1977) Biochim. Biophys. Acta 491, 296–304
10 Pownall, H.J., Hu, A., Gotto, A.M., Jr., Albers, J.J. and Sparrow, J.T. (1980) Proc. Natl. Acad. Sci. U.S.A. 77, 3154–3158
11 Jonas, A., Drengler, S.M. and Patterson, B.W. (1980) J. Biol. Chem. 225, 2183–2189
12 Dangreau, H., Joniau, M., De Cuyper, M. and Hanssens, I. (1982) Biochemistry 21, 3594–3598
13 Szoka, F. and Papahadjopoulos, D. (1978) Proc. Natl. Acad. Sci. U.S.A. 75, 4194–4198
14 Böhlen, P., Stein, S., Dairman, W. and Udenfriend, S. (1973) Arch. Biochem. Biophys. 155, 213–220
15 Vaskovsky, V.E., Kostetsky, E.Y. and Vasendin, I.M. (1975) J. Chromatogr. 114, 129–141
16 Schachman, H.K. (1959) Ultracentrifugation in Biochemistry, Academic Press, New York
17 Yi, P.N. and MacDonald, R.C. (1973) Chem. Phys. Lipids 11, 114–134
18 Watts, A., Marsh, D. and Knowles, P.F. (1978) Biochemistry 17, 1792–1801
19 Jones, A.J.S., Epand, R.M., Lin, K.F., Welton, D. and Vail, W.J. (1978) Biochemistry 17, 2301–2308
20 Van Cauwelaert, F., Hanssens, I., Herreman, W., Van Ceunebroeck, J.C., Baert, J. and Berghmans, H. (1983) Biochim. Biophys. Acta 727, 273–284
21 Hoff, H.F., Morrisett, J.D. and Gotto, A.M. (1973) Biochim. Biophys. Acta 296, 653–660
22 Segrest, J.P. (1977) Chem. Phys. Lipids 18, 7–22
23 Aune, K.C., Gallagher, J.G., Gotto, A.M. and Morrisett, T.D. (1977) Biochemistry 16, 2151–2156
24 Jonas, A., Drengler, S.M. and Kaplan, J.S. (1981) J. Biol. Chem. 256, 2420–2426
25 Schmidt, C.F., Lichtenberg, D. and Thompson, T.E. (1981) Biochemistry 20, 4792–4797
26 Jonas, A. and Drengler, S.M. (1980) J. Biol. Chem. 255, 2190–2196
27 Enoch, H.G., Fleming, P.J. and Strittmatter, P. (1977) J. Biol. Chem. 252, 5656–5660
28 Leto, T.L., Roseman, M.A. and Holloway, P.W. (1980) Biochemistry 19, 1911–1916
29 Tall, A.R. and Small, D.M. (1977) Nature 265, 163–164
30 Scherphof, G., Roerdink, F., Hoekstra, D., Zbrorowski, J. and Wisse, E. (1980) in Liposomes in Biological Systems (Gregoriadis, G. and Allison, A.C., eds.), pp. 179–209, John Wiley and Sons, New York