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Lipoprotein Models: Ultrasonicated Emulsions of Phosphatidylcholine, Cholesterol and Triolein

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A method for preparation of stable triolein–phosphatidylcholine and triolein–phosphatidylcholine–cholesterol emulsions is presented. The resulting emulsion particles are regarded as good models for structural and enzymatic studies of serum lipoproteins.

The maximal emulsifying effect was obtained at a w/w ratio of 2.2:1 between triolein and phosphatidylcholine, also in the presence of cholesterol. Emulsions constituted in this ratio proved to be rather homogeneous both in respect of particle size and lipid ratio. The cholesterol solubilizing capacity of the 2.2:1 triolein/phosphatidylcholine emulsion was found to be a cholesterol/phosphatidylcholine molar ratio of 2:1.

Electron microscopic pictures gave mean values for the particle diameters of 300 Å for a triolein-phosphatidylcholine, and 340 Å for a triolein-phosphatidylcholine-cholesterol emulsion. By light-scattering measurements the corresponding aggregate weights were found to be 0.8×10^7 and 1.3×10^7, respectively.

Cholesterol was found to have a condensing effect on egg phosphatidylcholine and to remove the gel-liquid crystal transition of dipalmitoyl phosphatidylcholine when added to 2.2:1 triolein/phosphatidylcholine emulsions in a 1:1 molar ratio with the phosphatidylcholine.

The results are discussed with respect to the structural arrangement of the lipid molecules and are found to be consistent with a particle with a nucleus of triolein and an interfacial region consisting of phosphatidylcholine and cholesterol.

The serum lipoproteins have a central role in lipid transport and metabolism. The relationship between the low density lipoproteins and the etiology of atherosclerosis is well recognized.[1,2] Additionally, the serum lipoproteins are valuable models for the elucidation of the principles of lipid-lipid and lipid-protein interactions.

The interactions between the main lipid classes in serum lipoproteins; phospholipids, cholesterol, cholesteryl esters, and triglycerides have been studied with different physicochemical methods.[3–8] The knowledge from such studies and investigations of intact lipoproteins [9–12] have led to a consistent structural model for the lipoprotein particle. The neutral lipids are assumed to be located in the particle core while the polar lipids together with the proteins would be organized in the interfacial region. Although these main principles are generally accepted there are several structural details which remain unsolved. One of the most intricate questions regards the explicit spatial arrangement of and interactions between the lipid components. It is difficult to study the features of molecular organization in an intact lipoprotein particle. Therefore we have chosen to use artificial models and compare them with native lipoproteins. By stepwise addition of each component it is possible to study the interaction between the separate components and their individual role in the final structural unit.

Another reason for preparation and investigation of lipoprotein models is their usefulness in enzymatic studies. There is a newly-awakened and justified interest in studying the influence of substrate structure on the enzyme activity of lipid-metabolizing enzymes and the mechanism of enzyme action.[13,14] In such studies it is essential to have substrates which resemble the natural ones, *e.g.*, lipoproteins and the properties of which can be varied in an appropriate way.

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MATERIALS AND METHODS

Materials. The egg phosphatidylcholine (EPC) used was prepared in this laboratory.[15] The molecular weight of it was 768 calculated from the fatty acid composition. The dipalmitoyl phosphatidylcholine (DPPC) was purchased from Sigma Chemical Co., St. Louis, MO. and used without further purification. The cholesterol was obtained from E. Merck, Darmstadt, Germany, and was recrystallized from 1,2-dichlorethane and found to be chromatographically pure by GLC and TLC. The triolein used was from Fluka AG, Buchs, Switzerland, and was purified by Florisil column chromatography. [^{14}C]Phosphatidylcholine was purchased from New England Nuclear Corp., Boston, MA. and [^{3}H]cholesterol and [^{3}H]triolein from Radiochemical Centre, Amersham, England. The purity of the radioactive compounds was checked by TLC. 8-Anilino-1-naphthalenesulfonic acid was obtained from Sigma Chemical Co., St. Louis, MO. Sepharose 2 B was purchased from Pharmacia Fine Chemicals, Uppsala, Sweden. Human serum low-density lipoproteins (LDL) were isolated by preparative ultracentrifugation. The buffer used was 0.1 M NaCl in 0.01 M barbital sodium pH 8.0.

Preparation of emulsions. The lipid mixtures were prepared by lyophilization from benzene solutions in thin-walled glass ampoules. After addition of buffer the ampoules were filled with N_2 gas and shaken. The ultrasonic irradiation was performed with a bath-type Branson Sonifier for 30 min at 25 °C for EPC preparations and at 45 °C for DPPC preparations. A rough suspension of triolein was first prepared by sonication. After addition of this to the phosphatidylcholine or phosphatidyl–choline–cholesterol mixture the sonication was completed. This procedure is important for obtaining proper emulsions.[16] No autoxidation or degradation during sonication could be detected by ultraviolet spectroscopy and thin layer chromatography.

Methods for characterization of the emulsions. Emulsions prepared by ultrasonication were subjected to molecular sieve chromatography on a Sepharose 2B column at 4 °C. The concentrations of lipids in the fractions were determined by liquid scintillation counting. Prior to light-scattering measurements the emulsions were filtered through a 0.05 μ Sartorius filter. The cholesterol solubilizing capacity of egg phosphatidylcholine-triolein emulsions was determined by sonication in the presence of excess cholesterol. Thereafter the emulsions were clarified by centrifugation at 50 000 *g* for 2 h at 4 °C and the proportions of lipids measured.

Negative staining electron microscopy was carried out by drying emulsions on Formvar-coated grids and staining with a 2 % solution of potassium phospohtungstate. The preparations were examined at once in an electron microscope. Light-scattering measurements were performed with a Sofica 4200 light-scattering photometer at 546 nm with the cell immersed in benzene at 25 °C.

Fluorescence measurements were made on a Perkin-Elmer 512 spectrophotometer. 8-Anilinonaphthalene-1-sulfonic acid (ANS) was used as a conformational sensitive fluorescent probe. The temperature of the sample chamber was controlled by means of a thermostatted bath. The actual temperature was checked directly in the cuvette. The ANS fluorescence was recorded at the emission maximum 480 nm with an excitation wavelength of 350 nm. The ANS concentration was 2×10^{-5} M and the dipalmitoyl phosphatidylcholine contents 2×10^{-4} M.

Proton nuclear magnetic resonance (^{1}H NMR) spectra were obtained with a JEOL FX-60 spectrometer operating in the Fourier transform mode. The NMR samples were thoroughly outgassed and the tubes filled with nitrogen and tightly stoppered. The temperature of the sample was controlled within 1 °C.

RESULTS

Optimal lipid ratios. Emulsions were prepared by ultrasonic irradiation of mixtures of egg phosphatidylcholine, cholesterol and triolein at different proportions of the lipids. The initial stoichiometry was found to influence the final particle composition and experiments were designed to find the optimal lipid ratios for obtaining proper emulsions.

If the concentration of egg phosphatidylcholine is increased, at a constant triolein (TO) concentration of 1 mg/ml, the optical density falls down to a minimum value at a ratio of 0.45 between EPC and TO (Fig. 1). The maximal emulsifying effect is thus obtained at a TO/EPC (w/w) ratio of 2.2:1. The present study is limited to preparations with TO/EPC ratios around this value.

When the emulsions were constituted in a TO/EPC ratio of 3:1 a rather inhomogeneous preparation was obtained. Negative electron microscopic pictures show particles with diameters ranging from 200 to 800 Å (Fig. 2). By molecular sieve chromatography the emulsion particles were separated according to their size (Fig. 3a). The fraction with the largest particles showed the highest TO/EPC values. The smaller particles had a rather constant TO/EPC value of ~ 2 (Fig. 3b).

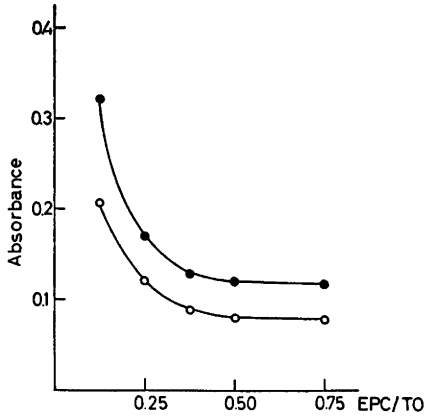

Fig. 1. The variation of the absorbance at 450 nm for TO-EPC (○) and TO-EPC-C (●) emulsions as a function of the EPC-TO weight ratio. The concentration of TO was held constant at 1 mg/ml and the molar ratio between EPC and C was 1:1.

Starting with a TO/EPC ratio of 1:1, a mixture of emulsion particles and phosphatidylcholine vesicles was obtained as stated by electron microscopy. Separated on the Sepharose 2 B column the preparation proved to be rather homogeneous regarding lipid ratio with somewhat more triolein (*i.e.* emulsion particles) among the large particles and phosphatidylcholine (*i.e.* vesicles) among the smaller ones (Fig. 3a, b).

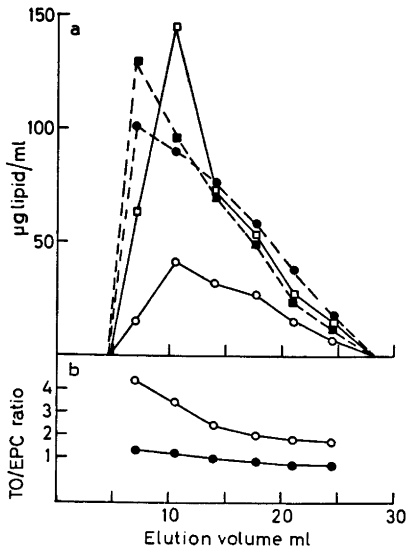

Fig. 3. a, Elution pattern of 3:1 and 1:1 TO/EPC emulsions with concentrations of TO (□,■) and EPC (○,●) in the fractions; b, the TO/EPC ratios in the corresponding fractions of the 3:1 (○) and the 1:1 (●) preparations.

Emulsions constituted in the 2.2:1 TO/EPC ratio, which was found to give maximal emulsification (see Fig. 1), were found to be rather homogeneous both in respect of particle size (Fig. 4) and lipid ratio (Fig. 5a, b). The particle size is in the same order of magnitude

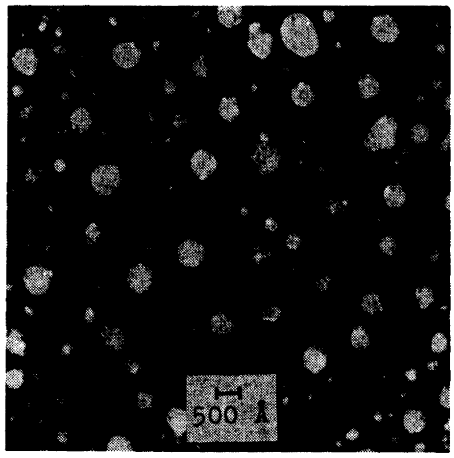

Fig. 2. Electron microscopic picture of 3:1 TO/EPC emulsion particles negatively stained with potassium phosphotungstate.

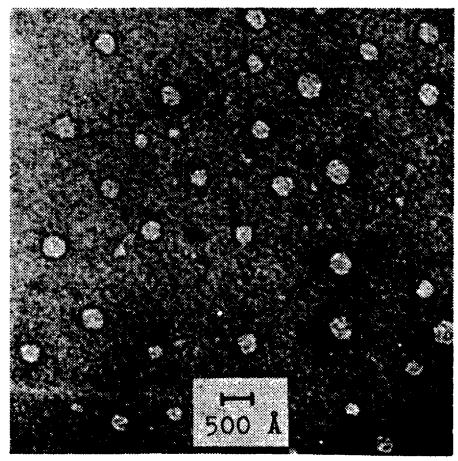

Fig. 4. Electron microscopic picture of negatively stained 2.2:1 TO/EPC emulsion particles.

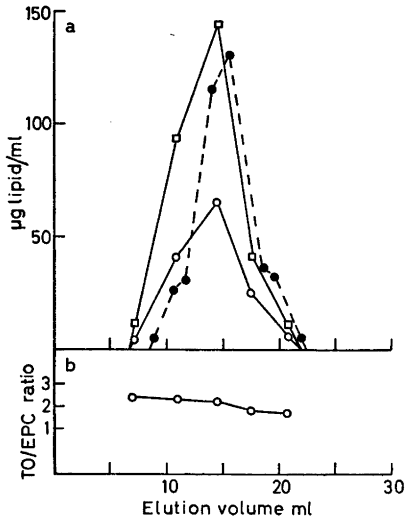

Fig. 5. a, Elution pattern of a 2.2:1 TO/EPC emulsion preparation with concentration of TO (□) and EPC (○) in the fractions (low density lipoprotein (●) as comparison); b, the TO/EPC ratios in the corresponding fractions.

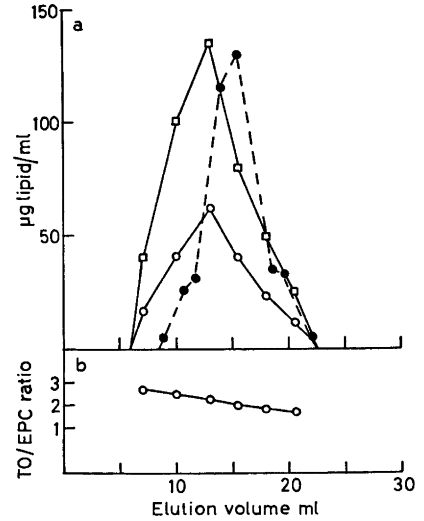

Fig. 6. a, Elution pattern of a TO–EPC–C emulsion with a TO/EPC ratio of 2.2:1 and an EPC/C mol ratio of 1:1. The concentrations of TO and EPC in the fractions are marked with □ and ○, respectively (low density lipoprotein (●) as comparison). The EPC/C mol ratio was 1:1 in all fractions; b, the TO/EPC ratios in the corresponding fractions.

as serum low-density lipoproteins. The diameter of the 2.2:1 emulsion particles was calculated as a mean value of 100 particles from an electron microscopic picture and gave 300 Å with extreme values at 200 and 400 Å.

From the curve of optical density *versus* EPC/TO ratio it can be seen that the maximal emulsification is obtained at the same EPC/TO ratio also in the presence of cholesterol (C) in a 1:1 mol ratio between EPC and C (Fig. 1). If an emulsion is constituted in a 2.2:1 TO/EPC ratio and with an EPC/C mol ratio of 1:1 a rather homogeneous preparation is obtained (Fig. 6a, b). The mean value for the diameter of the particles was calculated as 340 Å.

Characteristics of the emulsion particles. The apparent weight-average aggregate weights of egg phosphatidylcholine–triolein and egg phosphatidylcholine–cholesterol–triolein emulsions were measured by light-scattering measurements. The aggregate weight of low density lipoprotein was measured as comparison (Fig. 7). The values for the 2.2:1 TO/EPC emulsion and the same emulsion with cholesterol in a 1:1 molar ratio with egg phosphatidylcholine were found to be 0.8×10^7 and 1.3×10^7, respectively, and that of serum low density lipoprotein 0.4×10^7.

The ^{1}H NMR spectra of the 2.2:1 TO/EPC emulsion and the same emulsion with cho-

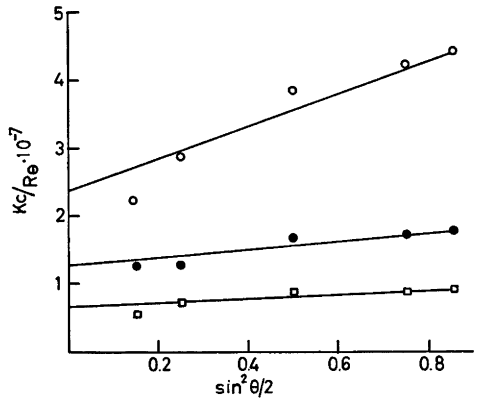

Fig. 7. Modified Zimm plots for emulsion preparations with 2.2:1 TO/EPC (●), and 2.2:1 TO/EPC with C in the same molar concentration as EPC (□) (low density lipoprotein (○) as comparison). An extrapolation of the lines to zero angle gives the reciprocal of the weight-average particle weights.

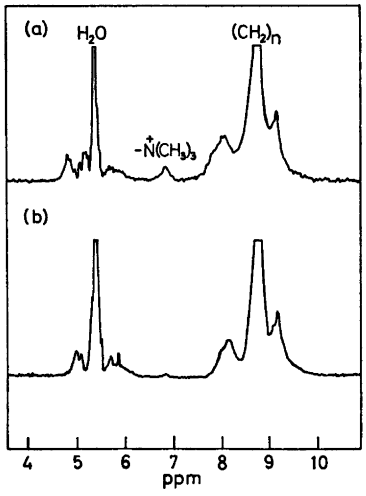

Fig. 8. High resolution ^1H NMR spectra of TO-EPC emulsion (a) and the same emulsion with C in a 1:1 molar ratio to EPC (b) both preparations dispersed in D_2O.

lesterol in a 1:1 EPC/C molar ratio are presented in Fig. 8. The most notable difference in the spectra is a broadening of the $N(CH_3)_3$ signal in the sample containing cholesterol.

The relative intensities of the ANS fluorescence as a function of temperature for dipalmitoyl phosphatidylcholine vesicles, dipalmitoyl phosphatidylcholine – triolein emulsion, and the same preparations with a 1:1 DPPC/C molar ratio of cholesterol are shown in Fig. 9. A sharp increase in the ANS fluorescence is observed at the gel-liquid crystal phase transition of dipalmitoyl phosphatidylcholine for both vesicles and emulsion. The samples containing cholesterol showed no such temperature dependent increase in fluorescence.

In order to clarify the distribution of cholesterol molecules between nucleus and interfacial region the cholesterol solubilizing capacity of the 2.2:1 TO/EPC emulsion was studied. The maximum value was found to be a C/EPC molar ratio of 2:1, the same value as for egg phosphatidylcholine vesicles at low lipid concentrations.[7]

The emulsions were found to be very stable. The increase in optical density was less than 10 % after a period of 1 month. The stability of emulsions with and without cholesterol was in the same order of magnitude.

Preliminary studies have shown that the emulsion preparations described herein are useful substrates for lipolytic enzymes.

DISCUSSION

In the present study the preparation of stable emulsions with a particle dimension close to that of serum low density lipoprotein is described. The lipid composition and ratio of these emulsions can be varied in a systematic way. Thus they provide a good model for studying molecular interaction in serum lipoproteins.

Studies of the system egg phosphatidylcholine – triolein – water have shown that EPC is very scarcely soluble in triolein (~ 1 %).[8] It is thus predictable that all the surface active phosphatidylcholine molecules are located in the interfacial region. Electron microscopic pictures of 2.2:1 TO/EPC emulsions show negligible presence of multilayers around the particles. It is evident that the phosphatidylcholine molecules are organized in a monolayer surrounding the particles.

Are there any triolein molecules in the inter-

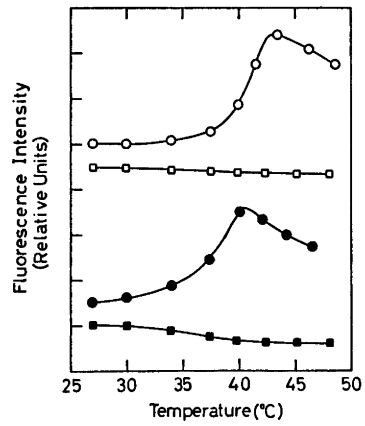

Fig. 9. Demonstration of the DPC gel-liquid crystal phase transition using ANS as a fluorescence probe. An increase in the ANS fluorescence is observed at the phase transition for the vesicle (○) and emulsion (●) preparations without C. If C is added in a 1:1 molar ratio to DPPC no increase is noted neither for vesicle (□) nor for emulsion particles (■).

facial region? An answer to this question can be achieved by combining the results from the present study with those of surface balance studies. Starting from a monolayer of phosphatidylcholine around a 2.2:1 TO/EPC emulsion particle with a diameter of 300 Å and taking the densities of egg phosphatidylcholine and triolein as 1.02 g/cm³ [15] and 0.90 g/cm³,[18] a surface area of 89 Å² can be calculated for the phosphatidylcholine molecules. X-Ray diffraction and surface balance studies of egg lecithin have given values of 58 Å²/molecule for the surface area at the fully hydrated state and the collapse point respectively.[19,20] A comparison with the value 89 Å²/phosphatidylcholine molecule in the surface film of the emulsion particle indicates that the phosphatidylcholine in this is in an expanded state. This is in agreement with a recent report [21] where a value of 80 Å²/molecule for phosphatidyl serine at an oil/water interface is calculated and it is predicted that phospholipids tend to be more expanded at the oil/water than at the air/water interface.

Surface balance measurements of mixed phosphatidylcholine – triolein films on a water surface have shown that triolein is ejected from the mixed monolayer at the collapse pressure of triolein. The two-dimensional solubility of triolein in lecithin was found to be very small.[20] It can be assumed that the conditions at the lipid-water interface of an emulsion particle are analogous to those in mixed monolayers on water. The conclusion is that the triolein molecules are located in the nucleus of the emulsion particle.

The distribution of cholesterol molecules between interfacial region and nucleus is another interesting detail. The cholesterol solubilizing capacity of egg phosphatidylcholine vesicles at low lipid concentrations is a C/EPC mol ratio of 2:1.[7] In the present study the same C/EPC mol ratio was found for the egg phosphatidylcholine – cholesterol – triolein emulsion particles. The bulk phase solubility of cholesterol in triolein at 25 °C is 3 %,[5] but the localization of the polar cholesterol molecules at the lipid-water interface is obviously more favourable than in the triolein nucleus. Therefore the most likely conclusion is that the cholesterol molecules are located in the interfacial region.

It is well-known from surface balance,[20] X-ray diffraction,[22] and spectroscopic [23] studies that cholesterol has a condensing effect on phosphatidylcholine, restricting the molecular motions. Obviously this is the case also in the mixed phosphatidyl choline – cholesterol monolayer around an emulsion particle. This can be deduced from Fig. 1 where the curves optical density *versus* EPC/TO ratio for phosphatidylcholine – triolein and phosphatidylcholine – cholesterol – triolein emulsions reach their minimum at the same EPC/TO ratio. The restricting effect of cholesterol is also noted in the ^{1}H NMR spectra (Fig. 8) where the broadening of the $N(CH_3)_3$ signal indicates a reduced mobility. Starting from a diameter of 340 Å for the egg phosphatidylcholine – cholesterol – triolein particle, taking the densities of egg phosphatidylcholine and triolein as above and that of cholesterol as 1.067 g/cm³,[18] and assuming a constant surface area of 37.5 Å² for cholesterol [10] a value of 53 Å² for the surface area of the egg phosphatidyl choline molecules can be calculated. This value is in good agreement with the partial molecular surface area of 54 Å² found by X-ray diffraction measurements on the lamellar phase of hydrated 1:1 molar mixtures of egg phosphatidylcholine and cholesterol.[22]

Besides the condensing effect, cholesterol has also been found to abolish the phosphatidylcholine gel – liquid crystal transition [23] or change it from a cooperative to a non-cooperative event [24] in bulk systems and vesicle preparations. The ANS fluorescence measurements (Fig. 9) show that this is valid also for the interaction between dipalmitoyl phosphatidyl choline and cholesterol in the interfacial region of an emulsion particle.

The lipid composition of the emulsions described here mostly resembles that of serum very-low-density lipoprotein, but obviously the principles of lipid – lipid interactions can also be adapted to other types of lipoproteins. Moreover the same methods can also be used for preparing emulsion particles containing cholesteryl esters.[16] In the future we also hope we will be able to add specific proteins to our lipid core models in order to study lipid – protein interactions.

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