Biochimica et Biophysica Acta, 513 (1978) 185—197
© Elsevier/North-Holland Biomedical Press

BBA 78164

INTERACTION OF CHOLESTEROL, PHOSPHOLIPID AND APOPROTEIN IN HIGH DENSITY LIPOPROTEIN RECOMBINANTS

ALAN R. TALL * and YVONNE LANGE

Division of Biophysics, Department of Medicine, Boston University School of Medicine, Boston, Mass. 02118 (U.S.A.)

(Received April 3rd, 1978)

Summary

To examine the effect of incorporation of cholesterol into high density lipoprotein (HDL) recombinants, multilamellar liposomes of ^{3}H cholesterol/^{14}C dimyristoyl phosphatidylcholine were incubated with the total apoprotein (apoHDL) and principal apoproteins (apoA-1 and apoA-2) of human plasma high density lipoprotein. Soluble recombinants were separated from unreacted liposomes by centrifugation and examined by differential scanning calorimetry and negative stain electron microscopy. At 27° C, liposomes containing up to approx. 0.1 mol cholesterol/mol dimyristoyl phosphatidylcholine (DMPC) were readily solubilized by apoHDL, apoA-1 or apoA-2. However, the incorporation of DMPC and apoprotein into lipoprotein complexes was markedly reduced when liposomes containing a higher proportion of cholesterol were used. For recombinants prepared from apoHDL, apoA-1 or apoA-2, the equilibrium cholesterol content of complexes was approx. 45% that of the unreacted liposomes. Electron microscopy showed that for all cholesterol concentrations, HDL recombinants were predominantly lipid bilayer discs, approx. 160 × 55 Å. Differential scanning calorimetry of cholesterol containing recombinants of DMPC/cholesterol/apoHDL or DMPC/cholesterol/apoA-1 showed, with increasing cholesterol content, a linear decrease in the enthalpy of the DMPC gel to liquid crystalline transition, extrapolating to zero enthalpy at 0.15 cholesterol/DMPC. The enthalpy values were markedly reduced compared to control liposomes, where the phospholipid transition extrapolated to zero enthalpy at approx. 0.45 cholesterol/DMPC. The calorimetric and solubility studies suggest that in high density lipoprotein recombinants cholesterol is excluded from 55% of DMPC molecules bound in a non-melting state by apoprotein.

---

* Present address: Department of Medicine, College of Physicians and Surgeons of Columbia University, The Presbyterian Hospital, 630 w. 168th Street, New York, N.Y. 10032, U.S.A.

Abbreviation: HDL, high density lipoprotein; DMPC, dimyristoyl phosphatidylcholine.

Introduction

Incubation or sonication of phosphatidylcholine multilamellar liposomes with the apoproteins of plasma high density lipoproteins (apoA-1, apoA-2, or apoHDL) * results in the incorporation of phospholipid into soluble lipoprotein complexes [1—3], which have been characterized as phospholipid bilayer discs by electron microscopy [4] and small angle X-ray scattering [5]. The stabilizing force of these complexes is primarily a hydrophobic interaction between apoprotein and lipid [6,7], which results in a conformational change of the apoprotein [8,3]. On the basis of studies of high density lipoprotein recombinants by scanning calorimetry and electron microscopy, Tall et al. [9] have suggested that the apoprotein forms an annulus around the perimeter of the phospholipid bilayer disc, protecting the hydrocarbon chains of the phospholipids from interaction with water. In this model there is an immobilized boundary layer of phospholipid 1—2 molecules thick, in contact with apoprotein at the edge of the disc, resulting in a decrease in enthalpy of the phospholipid gel to liquid crystalline transition.

Intrinsic membrane proteins, such as the cytochrome oxidase, or the proteolipid apoprotein are surrounded by an annulus of boundary phospholipid [11, 12]. Such proteins may modify the interaction of cholesterol with this boundary lipid. Cholesterol is excluded from the annulus of phospholipid surrounding the sarcoplasmic reticulum ATPase [13], and probably from the boundary lipid of some intrinsic red cell membrane proteins [14]. On the other hand, cholesterol is attracted to membrane pore-forming polyene antibiotics, such as amphotericin B, resulting in increased enthalpy of the phospholipid gel to liquid crystalline transition upon incorporation into a phospholipid/cholesterol membrane [15].

The presence of cholesterol in phospholipid liposomes results in a diminished incorporation of lipid into HDL recombinants [10]. The cholesterol/phospholipid ratio ** of the recombinants is reduced compared to the unreacted liposomes, suggesting exclusion of cholesterol from a fraction of recombinant phospholipid, possibly the boundary lipid [10]. In the present study we have found that for recombinants of DMPC with apoA-1, apoA-2 or apoHDL the cholesterol/phospholipid ratio of the recombinants is consistently approx. 45% of the unreacted liposomes and that the diminished formation of recombinants from cholesterol containing liposomes tends to parallel changes in the liposome permeability. To test further the cholesterol exclusion hypothesis, we have examined cholesterol containing recombinants by differential scanning calorimetry. Exclusion of cholesterol from the non-melting boundary phospholipid would result in a marked decrease in enthalpy of the DMPC gel to liquid crystalline transition compared to cholesterol-free recombinants; whereas non-exclusion of cholesterol would result in a less pronounced, predictable reduction in enthalpy.

---

* The total apoprotein of the plasma high density lipoprotein (apoHDL) consists of approximately 60% apoA-1 (M_r = 28 300), 30% apoA-2 (M_r = 17 380), and 10% C peptides (M_r = 7—8000) [1,31].

** Throughout this paper the proportions of cholesterol and phosphatidylcholine are given as a molar ratio.

Materials and Methods

Human plasma high density lipoproteins were prepared from the plasma of normal, fasting male donors by preparative ultracentrifugation between *d* 1.063 and 1.21 g/ml, and washed once at *d* 1.21 g/ml to eliminate albumin contamination. Lipoproteins were delipidated with chloroform/methanol (2 : 1, v/v) and washed three times with anhydrous diethyl ether at −15° C [17]. The composition of apoHDL, determined by Sephadex G-200 chromatography in 8 M urea, was 60% apoA-1, 30% apoA-2, and 10% C peptides. ApoA-1 was isolated from apoHDL by chromatography on Sephadex G-200 in 0.1 M Tris/8 M urea, pH 8.5, according to the method of Scanu et al. [18]. Eluates were monitored by ultraviolet absorption at 280 nm and urea and SDS polyacrylamide disc gel electrophoresis. Pure apoA-1 was obtained from the third peak. The fourth peak, containing apoA-2, was contaminated with apoA-1. Pure apo-A2 was obtained following dialysis, lyophilization and re-chromatography of the fourth peak on Sephadex G-200. Fractions containing pure apoproteins were pooled, dialyzed for 2—3 days against at least 4 changes of $(NH_4)_2CO_3$ buffer, pH 9.0, lyophilized and used within 20 days of preparation.

Dimyristoyl phosphatidylcholine was purchased from Serdary Company and purified to more than 99% purity by silicic acid chromatography. For preparation of ^{14}C-labeled DMPC, DMPC was digested with phospholipase D, by the method of Kornberg and McConnell [19], to form dimyristoyl phosphatidic acid, which was isolated by silicic acid chromatography. The dimyristoyl phosphatidic acid was coupled with ^{14}C-labeled choline (New England Nuclear) by the method of Aneja and Chadha [20], as modified by Sears et al. [21]. The resulting ^{14}C-labeled DMPC was isolated by silicic acid chromatography and was more than 99% pure by thin layer chromatography. In some centrifugation experiments, apoHDL was labeled with ^{125}I by the method of MacFarlane [22]. 99% of the ^{125}I counts were precipitable with 100% trichloracetic acid. Cholesterol of greater than 99% purity was purchased from Nu-Check Prep (Elysian, Minn.) and [^{3}H]cholesterol from New England Nuclear (Boston, Mass.). Cholesterol was assayed according to the technique of Parekh and Jung [23]. Phospholipid was assayed by phosphate determination [24].

For preparation of lipoprotein recombinants, aliquots of stock solutions in chloroform of [^{14}C]DMPC and [^{3}H]cholesterol of known specific activities were mixed in appropriate proportions. The solvent was evaporated under N_2 and the lipids lyophilized from benzene. The lipids were dispersed in buffer (0.01 M Tris/0.15 M NaCl/0.01% sodium azide, pH 8.0), by stirring at a temperature above the DMPC gel-liquid crystal transition temperature and then a solution of apoprotein was added. The mixtures were incubated at 27°C, except where otherwise noted, under N_2. In studies of the time course of recombinant formation, aliquots of the mxiture were centrifuged at 12 000 × *g* for 5 min in 0.4 ml conical plastic tubes in a Brinkmann model 3200 centrifuge. An aliquot of the supernatant was taken for the determination of radioactivity. The pellet was washed once and also analyzed for radioactivity. In some experiments the amount of lipid in the pellet was calculated from the total DMPC counts per min minus DMPC counts per min in the supernatant. The lipid composition of pellet and supernatant was determined from the

specific activities of [^{14}C]DMPC and [^{3}H]cholesterol. High density lipoprotein recombinants were also isolated by sequential preparative ultracentrifugation between *d* 1.07 and 1.21 g/ml, and by equilibrium density gradient ultracentrifugation at 10°C. Linear density gradients of NaBr were generated with a Pharmacia peristaltic pump and centrifuged for approx. $4.5 \cdot 10^6 \times g \cdot h$, in a Beckman SW56 swinging bucket rotor.

Differential scanning calorimetry. Differential scanning calorimetry was performed on a Perkin-Elmer DSC-2, as previously described [9]. 25—75-μl samples were hermetically sealed in 75-μl capacity stainless steel pans. Heating and cooling rates of 1.25—5°C/min were used and most experiments were performed close to full scale sensitivity (0.1—0.5 mcal/s). The instrument was calibrated with cyclohexane and indium standards, and with samples of DMPC in 25, 50, and 75 μl of buffer at heating rates of 0.31, 0.62, 1.25, 2.5, and 5°C/min. Transition temperatures were extrapolated to zero heating rate in order to determine temperature corrections for thermal lags in large volume samples. The maximum correction was 1.5°C for a 75 μl sample heated at 5°C/min. Enthalpy measurements were obtained from the area under thermal transitions, compared to the standard.

Electron microscopy. Lipoproteins were negatively stained with 2% sodium phosphotungstate, pH 7.4, on Formvar-coated copper grids. Electron micrographs were obtained with an AEI-6B electron microscope, calibrated with a catalase standard, at magnification approximately 100 000×. Samples containing about 1 mg/ml lipid were applied to the grids for 30 s, excess fluid was removed and then phosphotungstate applied for 15 s. For sizing of lipoproteins, at least three different fields from each of three different grids were employed with each preparation.

Results

Conditions of recombinant formation

The incorporation of lipid into recombinants was dependent on time of incubation, apoprotein identity, lipid/protein ratio, concentration of apoprotein, and temperature. The rate of formation of recombinants increased with decreasing lipid/protein ratio (Fig. 1a—c). Under similar conditions of temperature and apoprotein concentration, DMPC/apoHDL mixtures containing 86, 71 or 50% DMPC attained 50% of their equilibrium DMPC solubilization at approximately 4 h, 5 min and 2 min, respectively. A similar dependence on lipid/protein ratio was observed for cholesterol containing liposomes, but in the presence of cholesterol, the apparent equilibrium solubility was attained more rapidly (Fig. 1). This was also true for cholesterol containing recombinants of apoA-1 or apoA-2. Provided the initial DMPC/apoHDL ratio was 70/30 or less, the lipid solubilities obtained at 24 h for DMPC and DMPC/cholesterol liposomes, were not increased by extending incubations from 24 h to 3 days. At the same lipid:protein ratio, liposomes were solubilized a little more slowly in the presence of more concentrated solutions of apoHDL or apoA-1, especially at higher lipid/protein ratios. However, the incorporation of lipid into recombinants was complete at 23—46 h for mixtures of 70/30 DMPC/apoHDL or DMPC/apoA-1 using apoHDL or apoA-1 in either 0.5 or 4 mg/ml concentra-

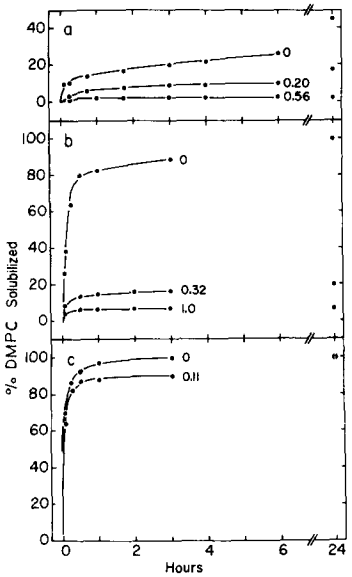


Fig. 1. Time course of solubilization of DMPC at 27°C for different DMPC/protein ratios. (a) 86/14, DMPC/apoHDL; (b) 71/29, DMPC/apoHDL; (c) 50/50, DMPC/apoHDL. In each case the mol ratio of cholesterol to DMPC initially present in the liposomes is identified. Recombinants were separated from unreacted lipid by centrifugation at 12 000 × *g* 5 min. This short centrifugal step was shown to give the same results for complex composition as preparative (*d* 1.07—1.21 g/ml) or equilibrium density gradient ultracentrifugation. In addition, liposomes incubated without apoHDL showed no appearance of phosphatidylcholine or cholesterol in the supernatant following centrifugation and less than 2% of counts of [^{125}I]apoHDL were recovered in the pellet of unreacted lipid.

tion. At the same lipid to protein ratio (70/30) solubilization of DMPC by apoapoA-2 was complete within 5 min, whereas solubilization by apoA-1 was only 50% completed at 12 h. The rate of solubilization of DMPC by apoHDL or mixtures of apoA-1 and apoA-2, was intermediate between that of apoA-1 and apoA-2, showing that apoA-2 facilitated the action of apoA-1. Below the gel to liquid crystalline transition temperature of the phosphatidylcholine, there was no incorporation of lipid into complexes. The extent of incorporation of DMPC and cholesterol into recombinants at 24 h was less at 40°C than at 27°C (Table I). In view of the above findings, the following conditions were chosen

TABLE I

DMPC SOLUBILIZED AT 24 h FOR MIXTURES CONTAINING DIFFERENT AMOUNTS OF CHOLESTEROL AT 27°C AND 40°C

mol cholesterol/mol DMPC in mixture	Fraction of DMPC solubilized	
	27°C	40°C
0	0.98	0.59
0.04	0.91	0.62
0.11	0.88	0.46
0.22	0.41	0.16
1.00	0.10	0.16

to determine the equilibrium lipid composition of recombinants; 70/30 DMPC/apoprotein ratio, incubation for 24 h at 27°C, apoprotein concentration, <2 mg/ml.

Lipid composition of cholesterol containing HDL recombinants

The presence of cholesterol in DMPC liposomes had a profound effect on the formation of HDL recombinants. At equilibrium liposomes containing up to about 0.1 cholesterol/DMPC were almost completely solubilized (Fig. 2). However, between 0.1 and 0.25 cholesterol/DMPC, there was a marked decrease in the extent of incorporation of lipid into complexes. Between 0.25 and 1.0 cholesterol/DMPC, about 5—30% of the phosphatidylcholine was solubilized. This abrupt decrease in solubility of lipid between 0.1 and 0.25 cholesterol/DMPC was observed for apoA-1, apoA-2, and apoHDL (Fig. 2), and was independent of lipid/protein ratio. Experiments performed with 0.25 and 0.43 cholesterol/DMPC liposomes showed no increase in DMPC solubility when lipid/protein weight ratio was varied from 2/1 to 1/20, while maintaining a constant apoprotein concentration. The decrease in DMPC solubility between 0.1 and 0.25 cholesterol/DMPC was observed at both 27 and 40°C (Table I).

The DMPC/cholesterol ratio of lipoprotein recombinants was markedly different from that of parent liposomes (Fig. 2b). The recombinants consistently contained 40—50% the cholesterol content of the unreacted liposomes

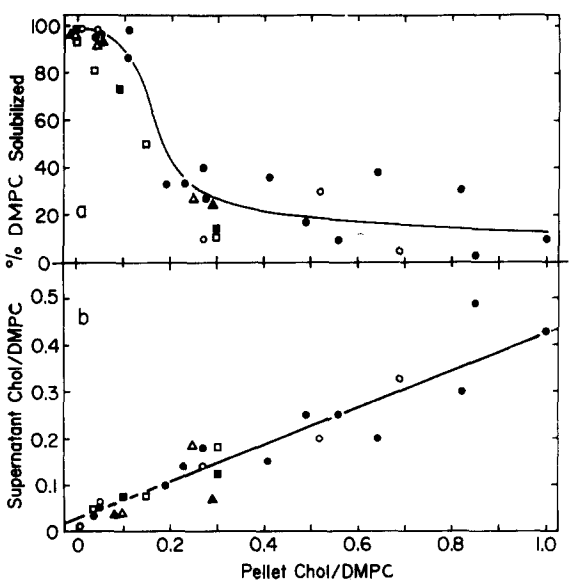


Fig. 2. Effect of liposome cholesterol content on equilibrium composition of recombinants containing various mol ratios of cholesterol (Chol). Liposomes (70/30, w/w, DMPC/apoHDL) were incubated with apoHDL (approx. 2 mg/ml), apoA-1, (0.2 mg/ml) or apoA-2 (0.2 mg/ml) for 24 h at 27°C. Upper curve shows % DMPC solubilized for the different mixtures. Lower curve gives the mol ratio of cholesterol in the recombinants plotted against the mol ratio in the liposomes at equilibrium. Recombinants were prepared as follows: closed circles, apoHDL, 12 000 × *g*, 5 min centrifugation; open circles, apoHDL, preparative ultracentrifugation between *d* 1.07 and 1.21 g/ml; closed squares, apoA-2, 12 000 × *g*, 5 min centrifugation; open squares, apoA-1, 12 000 × *g*, 5 min centrifugation; closed triangles, apoHDL equilibrium density gradient ultracentrifugation; open triangles, apoA-1, equilibrium density gradient ultracentrifugation. In b, the regression line was determined by the method of least squares.

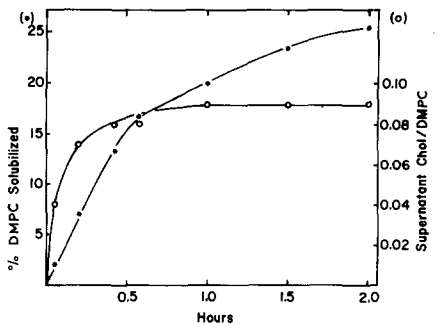


Fig. 3. Time course of solubilization of DMPC and cholesterol (Chol) from liposomes containing 0.25 cholesterol/DMPC. Recombinants were separated from unreacted lipid by centrifugation at 12 000 × *g* for 5 min.

(Fig. 2b). Thus, following completion of the incubation, unreacted liposomes were slightly enriched in cholesterol, while recombinant HDL was relatively cholesterol poor. At all molar ratios of cholesterol/DMPC, complexes formed earlier in the time course of the incubations contained less cholesterol than those formed later (see, for example, Fig. 3). However, the maximum cholesterol/DMPC ratio of the recombinants was always attained prior to the maximum solubilization of phosphatidylcholine.

Lipid/protein ratio

To determine the lipid/protein ratio of recombinants, mixtures containing 79/30 DMPC/apoHDL and 0, 0.55, 0.25, and 0.43 cholesterol/DMPC were incubated for 24 h at 27°C, and complexes isolated between *d* 1.07 and 1.21 g/ml. The DMPC/apoHDL weight ratios of these recombinants were, respectively, 70/30, 69/31, 66/34, and 74/26, indicating a constant lipid/protein ratio. When 0.055 cholesterol/DMPC liposomes were incubated for 24 h with apoHDL under conditions of excess lipid, i.e. at 70/30, 86/14, and 89/11 DMPC/apoHDL, the composition of the resulting complexes was 70, 77 and 77% respectively.

Electron microscopy

Negative stain electron microscopy showed that the complexes containing cholesterol were similar in appearance to those formed by DMPC/apoHDL, /apoA-1 or /apoA-2, i.e. phospholipid bilayer discs, which tended to form rouleaux of stacked discs, allowing discs to be view on edge (Fig. 4). Of the particles viewed on edge the predominant morphology was that of a lipid bilayer disc (about 160 × 55 Å). However, occasional particles had dimensions of approx. 240 × 110 Å, indicating that they were flattened vesicles. The circular particles were similar in diameter to the discs, suggesting they were discs laying flat on the grid. Identical structures were observed in the supernatants prepared from liposomes containing 0.11, 0.125, 0.43 and 1.0 cholesterol/DMPC, both early and late in the time course of recombinant formation. In contrast to recombinants prepared in the absence of cholesterol [9], no decrease in the diameter of discs could be achieved by decreasing the lipid to protein ratio from 70/30 to 40/60. When examined by electron microscopy,

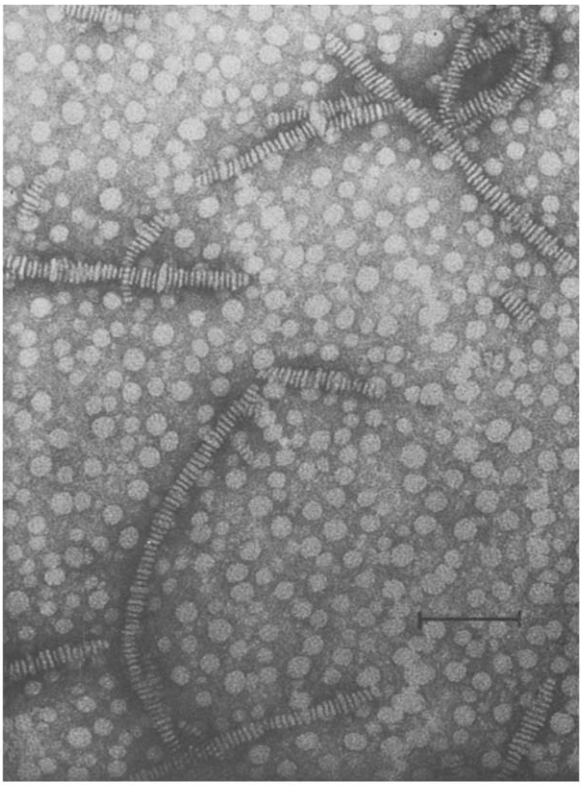


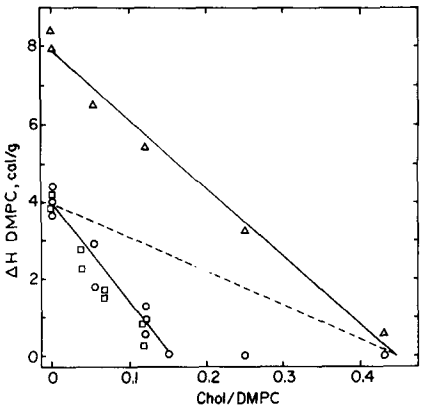
Fig. 4. Electron micrograph, negatively stained with sodium phosphotungstate, pH 7.4, of recombinants of DMPC/cholesterol (mol ratio, 0.11) with apoHDL. The bar on the micrograph indicates 1000 Å.

the pellet of unreacted lipid showed liposomes and no rouleaux of discs on edge.

Differential scanning calorimetry

(1) DMPC/cholesterol liposomes. Differential scanning calorimetry of cholesterol/DMPC liposomes gave results similar to those reported previously [25]. There was a linear decrease in enthalpy of the gel to liquid crystalline transition of the phospholipid from 8.3 cal/g for pure DMPC to 0.5 cal/g for 0.43 cholesterol/DMPC liposomes (Fig. 5). The enthalpy extrapolated to zero at approx. 0.45 cholesterol/DMPC. Identical enthalpy values were obtained heating at 1.25, 2.5 and 5°C/min.

(2) DMPC/cholesterol recombinants. Calorimetry was performed on recombinants prepared from DMPC/cholesterol liposomes and pure apoA-1 (Fig. 6) or apoHDL (not shown). Three types of transitions were observed; (a) the DMPC gel to liquid crystalline transition at about 27°C; (b) a high temperature transition at 70—80°C, representing denaturation of a lipoprotein complex, since it was not observed in preparations of pure phosphatidylcholine or apoprotein [9]; (c) a transition of peak temperature 54°C, representing the thermal denaturation of lipid-free apoA-1 [26], in supernatants prepared from more



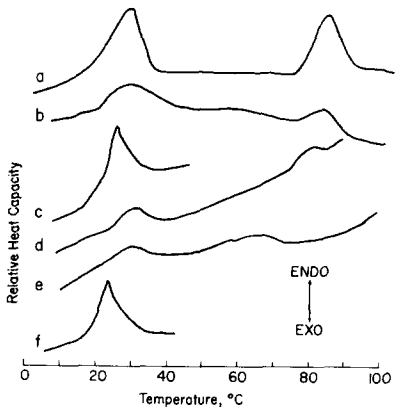


Fig. 5. Enthalpies of the DMPC gel to liquid crystalline transitions for cholesterol/DMPC liposomes (triangles), and DMPC/cholesterol/apoA-1 recombinants (squares). The ΔH values for recombinants containing 0.25 and 0.43 cholesterol/DMPC were calculated from data obtained on mixtures, given incorporation of lipid into recombinants similar to that shown in Fig. 2. The solid lines were determined by least squares regression analysis of the DMPC/cholesterol and DMPC/cholesterol/apoHDL experiments. Results obtained for DMPC/cholesterol/apoA-1 were not distinguishable from DMPC/cholesterol/apoHDL. The dotted lines shown the expected ΔH values for HDL recombinants in which cholesterol is randomly distributed throughout the phospholipid. The results for the recombinants are consistent with exclusion of cholesterol from a boundary layer of phospholipid (see Discussion).

Fig. 6. Differential scanning calorimetry of recombinants prepared from purified apoA-1 and liposomes of (a) 0, (b, c) 0.03, (d) 0.06 and (e, f) 0.11 mol cholesterol/mol DMPC. Mixtures were incubated at 27°C for 24 h and unreacted lipid separated from recombinants by low speed centrifugation. 75-μl aliquots of the supernatant solutions were examined in the DSC-2, at heating rates of 5°C/min, cooling rates of 20°C/min, at a sensitivity of 0.4 mcal/s. Each scanning pan contained approximately 0.8 mg DMPC and 0.4 mg apoA-1. Duplicate experiments were performed with each sample, giving thermograms similar to those shown. In a, b, d and e are shown the initial heating runs, while c and f show two heating runs obtained after initially heating through the high temperature endotherm. With heating and cooling between 0. and 50°C the initial phospholipid transition was entirely reversible. The different slopes of the baselines arise from variable instrument settings and do not denote sample variation. With increasing cholesterol content of the recombinants there was a marked decrease in enthalpy of the phospholipid gel to liquid crystalline transition and a shift to slightly higher temperature. Also, there was a decrease in the temperature and enthalpy of the high temperature endotherm arising from lipoprotein complex denaturation. In the 0.11 mol ratio preparation the peak at approx. 54°C indicates the presence of a small amount of lipid-free apoA-1.

than 0.15 cholesterol/DMPC liposomes (Fig. 6e).

Phospholipid transitions. Compared to control liposomes, the DMPC gel to liquid crystalline transition of recombinants was broadened and increased in temperature, as reported previously [9]. With increasing cholesterol content the DMPC gel to liquid crystalline transition of recombinants was decreased in enthalpy and at slightly higher temperature (Fig. 6, a, b, d and e). The sharp decrease in enthalpy with increasing cholesterol content was observed for recombinants prepared from both apoA-1 and apoHDL (Fig. 5). The decrease in enthalpy of the DMPC gel to liquid crystalline transition extrapolated to zero at approx. 0.17 cholesterol/DMPC (Fig. 5). After heating to 100°C there was a large increase in the enthalpy of the phospholipid transition (Fig. 6, c and f), which now resembled the transition of the control liposomes. If heated to 100°C these samples showed a denaturation endotherm of mid-point temperature 54°C (not shown). However, if the samples were left at 27°C for 1—2 h

their thermal behavior returned to that of the native recombinants, indicating a slow reassociation of lipid and apoA-1. For DMPC/apoHDL the reassociation was more rapid.

Free apoprotein transition. At 70/30 DMPC/apoA-1 ratio, supernatants prepared from liposomes of less than 0.11 cholesterol/DMPC showed no calorimetric evidence of free apoprotein, in agreement with the ultracentrifugation experiments. At mol ratios greater than 0.11 the 54°C transition due to free apoA-1 increased in enthalpy with increasing cholesterol content (Fig. 7).

Complex denaturation. Cholesterol containing recombinants showed a decrease in the temperature and enthalpy of the complex denaturation endotherm, compared to cholesterol-free recombinants (Fig. 7). These effects were more marked the greater the cholesterol content of the recombinant. Similar effects were observed for recombinants of both apoA-1 and apoHDL. For the mixtures shown in Fig. 7, the results are expressed as cal/g total apoHDL in mixture and, thus, the decrease in enthalpy partly reflects the presence of free apoA-1.

Effect of lipid/protein ratio

In all of the above experiments the ratio of DMPC to apoprotein was 70/30. To test the effect of varying lipid/protein ratio, differential scanning calorimetry was performed on supernatants prepared from 0.11 cholesterol/DMPC liposomes at different ratio of DMPC to apoHDL (70/30, 50/50, and 40/60, w/w). The enthalpy of the DMPC transition in the different preparations was constant, within the limits of experimental error. Also, the relative areas of the DMPC and complex denaturation endotherms were identical in the three supernatants, indicating the presence of a complex of invariant composition. A denaturation endotherm of peak temperature 54°C, indicating the presence of free apoA-1, was observed in the 50/50 and 40/60 supernatants.

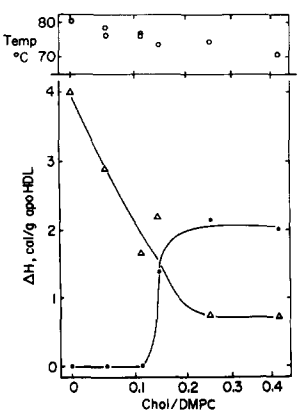


Fig. 7. Temperature (top), and enthalpy (bottom), of denaturation of DMPC/cholesterol/apoHDL recombinants as a function of mol ratio cholesterol/DMPC of the liposomes from which the recombinants were prepared. In the bottom panel, the circles represent the enthalpy of denaturation of lipid-free apoA-1 (peak temperature, 54°C).

Discussion

The findings of the present study confirm our preliminary observations [10] that the presence of cholesterol in phospholipid multilamellar liposomes inhibits the incorporation of lipid into high density lipoprotein recombinants. In the present study we have shown a marked decrease in solubility of cholesterol/DMPC liposomes between 0.1 and 0.2 mol cholesterol/mol DMPC, and a lesser incorporation of lipid into recombinants at 40°C than at 27°C. In a large number of preparations the molar ratio of cholesterol/DMPC recombinants was consistently 40—50% of unreacted liposomes (Fig. 2). The latter was true for both apoA-1 and apoA-2 and the mixture of apoproteins (apoHDL), and was confirmed by three different centrifugation techniques. That the mixtures had attained thermodynamic equilibrium was suggested by their invariant composition with further incubation, up to 3 days, and by the reversibility of phospholipid transitions when examined by differential scanning calorimetry.

The decreased incorporation of lipid into recombinants between 0.1 and 0.2 cholesterol/DMPC reflects the marked decrease in permeability of DMPC/cholesterol liposomes that occurs in this range [27]. Also, diminished incorporation of DMPC and cholesterol into complexes at 40°C compared to 27°C, may reflect the fact that permeability of liposomes reaches a maximum at the temperature of the phase transition. The importance of membrane permeability in recombinant formation suggests an initial stage of penetration of the membrane by the apoprotein, as is also implied by the proposed model of the discoidal recombinant [9]. During the process of membrane penetration, cholesterol is preferentially excluded from phospholipid in the immediate vicinity of the penetrating protein, especially early in the time course of recombinant formation (Fig. 2). Recombinant formation probably involves removal of a discoidal segment of phospholipid bilayer, as shown by nuclear magnetic resonance spectroscopic studies [28]. Following removal from the membrane, discoidal HDL may be come more enriched in cholesterol by exchange with liposomes or other lipoproteins.

Differential scanning calorimetry experiments were designed to test the possibility that the cholesterol-free phospholipid of recombinants (55%) comprises the boundary phospholipid. The reduced enthalpy of the DMPC gel to liquid crystalline transition of DMPC/apoHDL or DMPC/apoA-1 recombinants probably arises from non-melting of DMPC in the boundary layer [9]. Since 70/30 DMPC/apoHDL or DMPC/apoA-1 complexes have an enthalpy of approx. 4 cal/g DMPC, compared to a value of 8—9 cal/g for pure DMPC, 50—60% of the DMPC is in a boundary state [9]. Thus, in a lipoprotein complex containing 0.055 cholesterol/DMPC, the non-boundary lipid would be approx. 0.11 cholesterol/DMPC if the cholesterol were excluded from the boundary phospholipid, whereas it would be $\leqslant 0.055$ mol ratio if cholesterol were unaffected or attracted by apoprotein. The cholesterol exclusion hypothesis predicts an enthalpy of $5.5 \times 0.45 = 2.5$ cal/g DMPC for 0.55 mol ratio recombinants, since the ΔH of the 0.11 non-boundary lipid is 5.5 cal/g (Fig. 5) and this represents 45% of the DMPC in the recombinant. By similar reasoning, one would expect enthalpies of 1.5 cal/g for 0.12 and 0 cal/g for 0.20 mol ratio recombinants. For the same mol ratios, random distribution of cholesterol in

the phospholipid bilayer would result in enthalpies that were 40—50% of control liposomes, with extrapolation to sero enthalpy at approx. 0.45 cholesterol/DMPC (see dashed line, Fig. 5).

The experimental data (Fig. 5) is consistent with exclusion of cholesterol from the boundary layer of phospholipid in contact with apoprotein. The later represents about 55% of the phospholipid of 160 Å diameter discoidal HDL [9], which corresponds to the percentage of cholesterol-free phospholipid indicated by the compositional data (Fig. 2). Thus, cholesterol in the non-boundary phospholipid (40—50%) is in equilibrium with cholesterol in liposomes, while cholesterol is excluded from the boundary lipid. Although we have presented only limited compositional data on apoA-2/DMPC/cholesterol recombinants (Fig. 2), there was no major difference from recombinants of apoA-1 or apoHDL, suggesting that apoA-2 may also have a boundary layer of phospholipid which excludes cholesterol.

The concept of a phospholipid boundary layer, in which the melting of hydrocarbon chains is inhibited by interaction with apoprotein, is supported by recent studies of Stoffel et al. [29], who cross-linked 18-azidolinoleic acid-substituted phosphatidylcholine with apoA-1 in discoidal HDL recombinants. 44% of the phosphatidylcholine underwent cross-linking, 24% directly to apoA-1 and 17% to apoA-1 via other phosphatidylcholine molecules. The latter values may represent the percentage of phospholipid, respectively, in the outer and next to outer molecular layers of the discs, suggested by Tall et al. [9] to constitute the boundary lipid. The reduced enthalpy of the DMPC transition in the boundary layer may reflect a disordering of DMPC molecules below the gel to liquid crystalline transition and an ordering above the transition. Both effects were suggested by fluorescence studies of DMPC/apoA-1 recombinants [30]. Thus, apoA-1 and apoA-2 may exert an ordering/disordering effect on phospholipid molecules which is additive to that of cholesterol in HDL recombinants.

High density lipoprotein is probably secreted as a phospholipid bilayer disc by rat liver [32] and small intestine [33]. The limited solubility of unesterified cholesterol in recombinant HDL suggests that nascent HDL may have a limited capacity for dissolving unesterified cholesterol and that the cholesterol transporting capacity of HDL is greatly increased by the conversion of nascent HDL into cholesterol ester containing spherical HDL. Chylomicron phospholipid and apoA-1 probably also contribute to plasma HDL: during lipolysis of the chylomicrons there appears to be generation of phospholipid membranes at the lipoprotein surface [34], which may dissociate as phospholipid/apoA-1 discs or vesicles [35]. The increase in cholesterol/phospholipid ratio of chylomicron and very low density lipoprotein remnants [36] may result in part from relative exclusion of cholesterol from the dissociating particles.

Acknowledgements

We wish to thank Drs. Atkinson, Curatolo, Sears, Shipley and Small for their helpful criticisms of the manuscript, and to acknowledge the excellent technical assistance of Ms. V. Varner and L. Askinazi. This work was supported by NIH grants HL20303, GRS 605711, and HL18623. While part of this work was

being performed, Alan Tall was a Fellow of the Medical Foundation, Inc., Boston, Mass.

References

1 Morrisett, J.D., Jackson, R.L. and Gotto, A.M. (1975) Annu. Rev. Biochem. 44, 183—207
2 Assmann, G. and Brewer, H.B. (1974) Proc. Natl. Acad. Sci. U.S. 71, 989—993
3 Tall, A.R., Small, D.M., Shipley, G.G. and Lees, R.S. (1975) Proc. Natl. Acad. Sci. U.S. 72, 4940—4942
4 Forte, T.M., Nichols, A.V., Gong, E.L., Lux, S. and Levy, R.I. (1971) Biochim. Biophys. Acta 248, 381—386
5 Atkinson, D., Smith, H.M., Dickson, J. and Austin, J.P. (1976) Eur. J. Biochem. 64, 541—547
6 Stoffel, W., Zierenberg, O., Tunggal, B. and Schreiber, E. (1974) Proc. Natl. Acad. Sci. U.S. 71, 3696—3700
7 Finer, E.G., Henry, R., Leslie, R.B. and Robertson, R.N. (1975) Biochim. Biophys. Acta 380, 320—337
8 Lux, S.E., Hirz, R., Shrager, R.I. and Gotto, A.M. (1972) J. Biol. Chem. 247, 2598—2606
9 Tall, A.R., Small, D.M., Deckelbaum, R.J. and Shipley, G.G. (1977) J. Biol. Chem. 252, 4701—4711
10 Tall, A.R. and Lange, Y. (1978) Biochem. Biophys. Res. Commun. 80, 206—212
11 Jost, P.C., Griffith, O.H., Capaldi, R.A. and Vanderkooi, G. (1973) Proc. Natl. Acad. Sci. U.S. 70, 480
12 Curatolo, W., Sakura, J.D., Small, D.M. and Shipley, G.G. (1977) Biochemistry 16, 2313—2319
13 Warren, G.B., Houslay, M.D., Metcalfe, J.C. and Birdsall, N.J.M. (1975) Nature 255, 684—687
14 Bieri, V.G. and Wallach, D.F.H. (1975) Biochim. Biophys. Acta 406, 415—423
15 Norman, A.W. Demel, R.A., de Kruijff, B., Geurts van Kessel, W.S. and van Deenen, L.L.M. (1972) Biochim. Biophys. Acta 290, 1—14
16 Sodhi, H.S. and Gordon Gould, R. (1967) J. Biol. Chem. 242, 1205—1210
17 Scanu, A.M. and Edelstein, C. (1971) Anal. Biochem. 44, 576—588
18 Scanu, A., Toth, J., Edelstein, C., Koga, S. and Stiller, E. (1969) Biochemistry 8, 3309—3316
19 Kornberg, R.D. and McConnell, H.M. (1971) Biochemistry 10, 1111
20 Aneja, R. and Chadha, J.S. (1971) Biochim. Biophys. Acta 248, 455—253
21 Sears, B., Hutton, W.C. and Thompson, T.E. (1976) Biochemistry 15, 1635—1639
22 MacFarlane, A.S. (1958) Nature 182, 53
23 Parekh, A.C. and Jung, D.H. (1970) Anal. Chem. 42, 1423—1427
24 Gomori, G. (1942) J. Lab. Clin. Med. 27, 955
25 Hinz, H. and Sturtevant, J.M. (1972) J. Biol. Chem. 247, 3697—3700
26 Tall, A.R., Shipley, G.G. and Small, D.M. (1976) J. Biol. Chem. 251, 3749—3755
27 Blok, M.C., van Deenen, L.L.M. and de Gier, J. (1977) Biochim. Biophys. Acta 464, 509—518
28 Andrews, A.L., Atkinson, D., Barratt, M.D., Finer, E.G. Hauser, H., Henry, R., Leslie, R.B., Owens, N.L., Phillips, M.C. and Robertson, R.N. (1976) Eur. J. Biochem. 64, 549—563
29 Stoffel, W., Darr, W. and Salm, K. (1977) Hoppe-Seyler's Z. Physiol. Chem. 358, 453—462
30 Jonas, A., Krajnovich, D.J. and Patterson, B.W. (1977) J. Biol. Chem. 252, 2200—2205
31 Baker, H.N., Delahunty, T., Gotto, A.M. and Jackson, R.L. (1974) Proc. Natl. Acad. Sci. U.S. 71, 3631—3634
32 Hamilton, R.L., Williams, M.C., Fielding, C.J. and Havel, R.J. (1976) J. Clin. Invest. 58, 667—680
33 Green, P.H.R., Tall, A.R. and Glickman, R.M. (1978) J. Clin. Invest. 61, 528—534
34 Blanchette-Mackie, E.J. and Scow, R.D. (1976) J. Lipid Res. 17, 57—67
35 Tall, A.R. and Small, D.M. (1978) submitted for publication to N. Engl. J. Med.
36 Mjos, O.D., Faergeman, D., Hamilton, R.L. and Havel, R.J. (1975) J. Clin. Invest. 56, 603—615