Interactions of Dipalmitoyl- and Dimyristoylphosphatidylcholines and Their Mixtures with Apolipoprotein A-I†

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ABSTRACT: Human and bovine A-I apolipoproteins were incubated with multibilayer liposomes of dimyristoylphosphatidylcholine (DMPC) and dipalmitoylphosphatidylcholine (DPPC) and several mixtures of these two lipids. The reactions were carried out at temperatures around the transition temperature of the lipids, and the formation of small, micellar complexes of protein with lipid was followed as a function of time. Micellar complexes were isolated by ultracentrifugation and were characterized in terms of stoichiometry, lipid composition by gas chromatography, approximate size by gel filtration, and phase transition behavior by fluorescence polarization measurements. The results indicate a decrease in reaction rates with increasing DPPC contents of the mixtures, consistent with the higher stability of DPPC bilayers. Reactions have optimal rates at the transition temperature and are limited to the temperature range where gel and liquid-crystalline phases coexist. The isolated complexes with DMPC and DPPC have similar molecular weights in the range from 2×10^5 to 2.5×10^5, but lipid/protein mole ratios differ by about 40%. The lower lipid/protein ratio of DPPC complexes (100:1 mol/mol) is compensated by the longer acyl chains of this lipid, such that the acyl chain area of both complexes stabilized by apolipoprotein is essentially identical.

Interactions between apolipoprotein A-I (apo A-I)[1] and phospholipids determine the structure of high-density lipoproteins (HDL) and play a fundamental role in the biogenesis of these particles (Tall & Small, 1980).

Studies in the past few years have concentrated on the interactions of apo A-I with dimyristoylphosphatidylcholine (DMPC). They demonstrated that DMPC multibilayer liposomes and single bilayer vesicles react optimally with apo A-I at the transition temperature of the lipid (Pownall et al., 1978) to form discoidal, micellar complexes having molecular weights around 2×10^5 and weight ratios of 2–3 g of DMPC/g of apo A-I (Atkinson et al., 1976; Jonas et al., 1977; Tall et al., 1977). At very high ratios of lipid to protein, relatively stable vesicles of DMPC with bound apo A-I can be produced. These vesicular complexes can incorporate several monomers of apo A-I before they break up to give the final micellar complexes in a slow process (Jonas et al., 1980, 1981; Jonas & Drengler, 1980). Thus, the formation of micellar complexes is markedly affected by the concentration of apo A-I on the bilayer surface and by the physical state of the lipid.

Since the phase behavior of the lipid is of such importance in the DMPC interaction, we set out in this work to explore in detail the effects of adding a higher melting phospholipid, dipalmitoylphosphatidylcholine (DPPC), to DMPC on the reaction rates and products formed with apo A-I. During the course of our studies, a report was published by Swaney (1980a,b) on the interaction of apo A-I with DMPC mixtures with distearoylphosphatidylcholine and palmitoyloleoylphosphatidylcholine in terms of the phase behavior of the lipid mixtures, and the properties of some complexes of apo A-I with mixtures of DMPC plus DPPC or distearoylphosphatidylcholine. Our work extends these observations to complexes of apo A-I and pure DPPC which had not been previously characterized, establishes that reactions of apo A-I with lipid mixtures take place over the entire phase diagram region of gel and liquid-cryatalline phase coexistence, and describes the phase transition behavior of isolated complexes.

Experimental Procedures

Materials and Preparations. DMPC and DPPC were purchased from Sigma Chemical Co. By thin-layer chromatography silica gel plates developed in chloroform–methanol–H_2O (65:25:5 v/v) and detected by acid spray and charring, the lipids were >98% pure. ^{14}C-Labeled DPPC was obtained from New England Nuclear; this preparation was found to be >97% pure by the same TLC procedure as described above but using autoradiography for detection.

Human and bovine apo A-I's were prepared as described in the literature (Edelstein et al., 1972; Jonas, 1975) with some modifications (Jonas et al., 1980). By polyacrylamide gel electrophoresis in 0.01% sodium dodecyl sulfate and 7.5% acrylamide, both proteins showed a single band up to 50 μg of protein loading on the gels. Apo A-I preparations were stored at −20°C in lyophilized form. Before use, the proteins were dissolved in 5 M guanidine hydrochloride and were dialyzed extensively against buffer. All the experiments described in this work were performed in 0.1 M Tris-HCl, pH 8.0, 0.001% EDTA, and 10^{-3} M NaN_3 buffer. Protein concentrations were determined from published extinction coefficients (Jonas et al., 1977) or by using a modified Lowry assay (Markwell et al., 1978).

Phosphatidylcholine (PC) mixtures were prepared by weighing out the appropriate amounts of DMPC and DPPC to give samples ranging from 100 mol % DPPC to 100 mol % DMPC in 20 mol % increments. The fatty acid compositions of the PC mixtures measured by gas chromatography (GC) (Varian Series 3700 GC) coincided within ±5% with those determined by weighing. Up to 30 mg of PC plus about 2×10^6 cpm of [^{14}C]DPPC was mixed in chloroform and then was dried under N_2. Multibilayer liposomes were prepared by vortexing the lipids at temperatures >43 °C in 2.0 mL of buffer containing 5% KBr, intended to keep the lipid particles in suspension. Concentrations of PC were determined from

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[1] Abbreviations used: apo A-I, major protein component of HDL; HDL, high-density serum lipoprotein; DMPC, dimyristoylphosphatidylcholine; DPPC, dipalmitoylphosphatidylcholine; PC, phosphatidylcholine; DPH, diphenylhexatriene fluorescence probe; T_m, phase transition temperature of PC's.

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the ^{14}C counts and the specific activity of the preparations or by the procedure of Chen et al. (1956).

Reaction mixtures were prepared at room temperature to give a mole ratio of 150 PC/apo A-I and a protein concentration of 1.8×10^{-5} M. The buffer used in the preparation of the 2.0-mL samples contained 5% KBr. Incubations of samples at each lipid composition were performed at six different temperatures, regulated within ±0.1 °C in the vicinity of the phase transition temperature (T_m) of the particular PC composition (Lentz et al., 1976; Mabrey & Sturtevant, 1976). Samples were mixed, and 0.2-mL aliquots were removed periodically for analysis. The aliquots were centrifuged at 80 000 rpm in a Beckman airfuge for 10 min, at ambient temperature. These conditions were shown to sediment completely multibilayer PC dispersions and not to sediment to any significant extent micellar complexes of PC and apo A-I. After centrifugation, 0.1 mL of the supernatant was counted for ^{14}C counts per minute. The percent yield of PC in micellar complexes was determined from the maximum possible solubilization of PC, taking into account the stoichiometry of the various complexes. For example, for the 40 mol % DPPC complex, $^{125}/_{150}$ of all the PC in the sample appearing in the supernatant corresponds to 100% yield.

Isolation and Characterization of Complexes. Isolation of complexes was accomplished from samples which were incubated at the T_m of the lipid, for 20 h. The reaction mixtures were centrifuged at 18 000 rpm, at 15 °C, for 1 h to remove unreacted, multibilayer lipid. Analysis of these samples by gel filtration on Sepharose CL-4B columns indicated the absence of free lipid but considerable amounts of free apo A-I, particularly in the samples containing high levels of DPPC. The complexes were separated from free protein by ultracentrifugal flotation in a Beckman L5-65 ultracentrifuge at a density of 1.180 g/mL adjusted with NaBr. Centrifugation at 50 000 rpm, 15 °C, for 24 h was sufficient to separate completely the complexes from free apo A-I.

The stoichiometry of the complexes was determined after dialysis of samples against buffer, by measuring the absorbance at 280 nm and ^{14}C counts per minute, or by the modified Lowry assay for protein (Markwell et al., 1978) and the Chen et al. (1956) procedure for phosphate determination. The molecular weights of the complexes were estimated from gel filtration on a calibrated column of Bio-Gel A-5m (1.8 × 50 cm) with bovine HDL (380 000), apo A-I·DMPC micellar complexes prepared from DMPC vesicles (230 000) (Jonas et al., 1977), aldolase (158 000), bovine serum albumin (67 000), and ovalbumin (45 000) as the standards. The PC composition was determined by GC of fatty acid methyl esters (Metcalfe et al., 1966) prepared from the lipids extracted by the procedure of Folch et al. (1957) from the isolated complexes.

The phase transition determinations on PC bilayers and lipoprotein particles with the 1,6-diphenyl-1,3,5-hexatriene (DPH) fluorescent probe (Aldrich Chemical Co.) have been described previously (Shinitzky & Barenholz, 1974; Lentz et al., 1976; Jonas et al., 1980). Fluorescence polarization measurements were performed with an SLM Series 400 polarization spectrofluorometer, using 366-nm exciting light, Corning glass 3-74 filters, and 4-nm slits. Temperature was regulated to ±0.1 °C with a Forma Scientific circulating water bath.

Results

All the experiments to be described in this section were performed with human and bovine apo A-I. The results were essentially identical for both proteins, with the exception of the kinetics experiments where the rates of reaction with the human protein were 20–30% higher than those with bovine apo A-I.

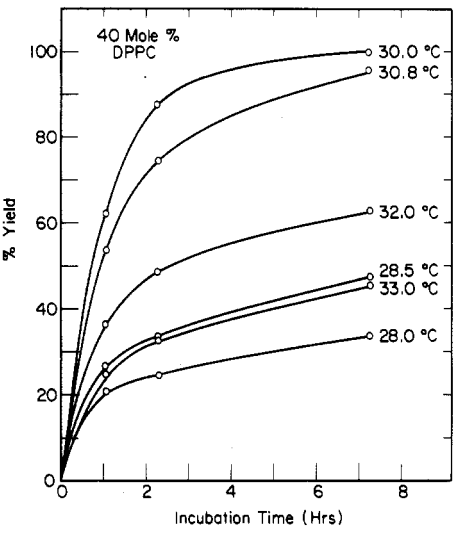

FIGURE 1: Percent yield of micellar complexes as a function of time for a reaction mixture containing 40 mol % DPPC and 60 mol % DMPC. The bovine apo A-I concentration was 1.8×10^{-5} M, and the PC/apo A-I mole ratio was 150:1.

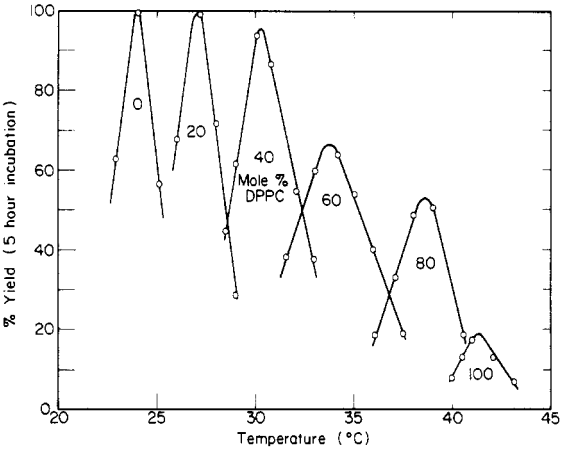

FIGURE 2: Percent yield of micellar complexes, after 5 h of incubation, as a function of temperature. From left to right, the mole percent of DPPC in the reaction mixtures was 0, 20, 40, 60, 80, and 100.

The reaction kinetics of a PC mixture containing 40 mol % DPPC and bovine apo A-I are shown in Figure 1. The rates of micellar product formation are highest at temperatures near the T_m of the lipid mixture (31.5 °C) and descrease markedly at either side of this temperature. Similar results were obtained with all the other PC compositions.

From the experiments on reaction kinetics, percent yields were obtained at a fixed time and were plotted as a function of temperature. Figure 2 shows the temperature dependence of the yields of reactions, after 5 h, for all the lipid mixtures examined. It is evident that in all cases optimum yields are attained at the T_m of each mixture and also that the yields decrease progressively with the content of the longer chain PC, DPPC. The temperature dependence of the yields is sharpest for the pure lipids and becomes broader for the intermediate lipid compositions. Initial reaction rates and the times required to reach equilibrium are summarized in Table I. They were estimated from plots such as that shown in Figure 1.

In Table II, we summarize the transition temperatures (T_m) for DPPC–DMPC mixtures obtained by differential scanning calorimetry (Mabrey & Sturtevant, 1976), by fluorescence polarization of DPH (Lentz et al., 1976; this work, Figure 3), and by observing optimum reaction rates with apo A-I (Figure

Table I: Initial Reaction Rates and Equilibration Times for Bovine Apo A-I Reactions with PC's, Carried Out at the Corresponding T_m Values

PC composition (mol % DPPC)	initial rate[a] (% yield/h)	equilibration time[b] (h)
0	148	3
20	96	5
40	60	8
60	38	24
80	19	>40
100	7.8	nd[c]

[a] Taken from the initial linear portion (0.25–1 h) of percent yield vs. time plots (e.g., see Figure 1). [b] Time required to reach equilibrium, estimated from percent yield vs. time plots (e.g., see Figure 1). [c] nd, not determined.

Table II: Phase Transition Temperatures for DMPC-DPPC Multibilayer Liposomes Determined by Different Methods

PC composition (mol % DPPC)	T_m (°C) DSC[a]	fluorescence polarization[b]	apo A-I reaction[c]
0	23.9	23.8	23.9
20	27.1	26.6	27.1
40	30.4	30.0	30.3
60	33.8	33.7	33.8
80	37.4	37.1	37.9
100	41.4	41.5	41.6

[a] From Mabrey & Sturtevant (1976). [b] From Figure 3. [c] From Figure 2 and related experiments.

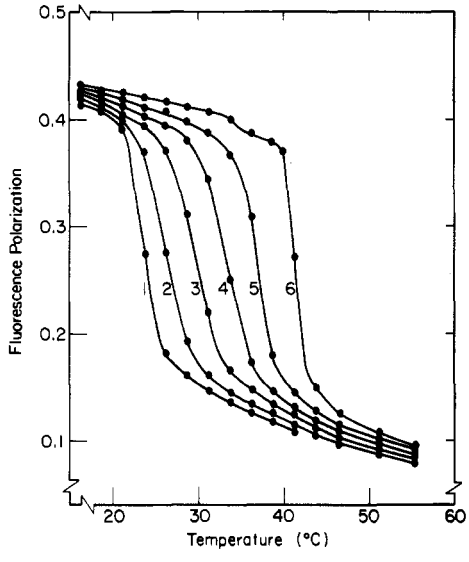

FIGURE 3: Phase transition behavior of DMPC (1) and DPPC (6) and their mixtures: 20 mol % DPPC (2), 40 mol % DPPC (3), 60 mol % DPPC (4), and 80 mol % DPPC (5). The fluorescence polarization of DPH, dissolved in multibilayer liposomes in mole ratios of 500:1, PC/DPH, was measured as a function of temperature.

2). The agreement in all cases is very good.

The complexes of apo A-I with DPPC–DMPC mixtures isolated by centrifugation, at a density of 1.180 g/mL at 15 °C, remained stable for at least 2 weeks at 4 °C, as judged by the coelution of lipid and protein in a single peak from a Bio-Gel A-5m column. The stored complexes eluted at the same position and had the same composition as that of the original sample.

The elution positions of the various complexes from a calibrated Bio-Gel A-5m column were similar and corresponded to molecular weights in the range of (2–2.5) × 10^5, assuming particles of low asymmetry and hydration similar

Table III: Properties of Isolated Complexes of Apo A-I and PC Mixtures

PC composition of liposomes (mol % DPPC)	complexes PC/apo A-I[a] (mol/mol)	PC composition[b] (mol % DPPC)	T_m[c] (°C)
0	138 (±15%)[d]	0	27.1 (±3%)
20	130	24 (±10%)	30.6
40	125	42	34.1
60	122	62	38.3
80	117	81	42.4
100	100	100	45.1

[a] Average of five determinations. [b] From GC. [c] From Figure 4. [d] Percent errors are estimated experimental errors.

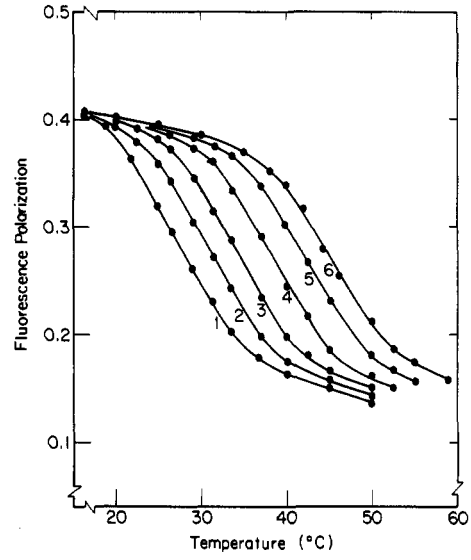

FIGURE 4: Phase transition behavior of isolated complexes of apo A-I with DMPC (1) and DPPC (6) and their mixtures: 20 mol % DPPC (2), 40 mol % DPPC (3), 60 mol % DPPC (4), and 80 mol % DPPC (5). The fluorescence polarization of DPH, dissolved in micellar complexes in mole ratios from 200:1 to 500:1, PC/DPH, was measured as a function of temperature.

to the standard proteins. The PC/apo A-I mole ratios of the complexes increase with the content of DMPC, from a mole ratio of 100:1 with DPPC alone to 140:1 with DMPC alone. On the basis of stoichiometry and the approximate molecular weights, each complex appears to contain two apo A-I polypeptide chains, in agreement with Swaney's (1980b) apo A-I cross-linking studies on his small complexes. The PC composition of the complexes, determined by GC, was identical, within experimental error, with that of the original PC mixtures. Stoichiometries, PC composition, and the phase transition temperatures for the isolated complexes are summarized in Table III.

Figures 3 and 4 show the phase transition behavior of DPPC–DMPC mixtures in multibilayer liposome form and in isolated complexes, respectively. Fluorescence polarization of DPH, plotted as a function of temperature, undergoes a relatively sharp change in the T_m region of DMPC liposomes (23.8 °C) and of DPPC liposomes (41.4 °C). The mixtures of both lipids, in liposome form, have intermediate T_m values and somewhat broader transitions (Figure 3). The isolated complexes have detectable phase transitions, but they are shifted 3–4 °C to higher temperatures and are about twice as wide as the phase transitions of the original lipid mixtures (Figure 4).

The results of the phase transition behavior of the liposomes and complexes are summarized on the phase diagram shown

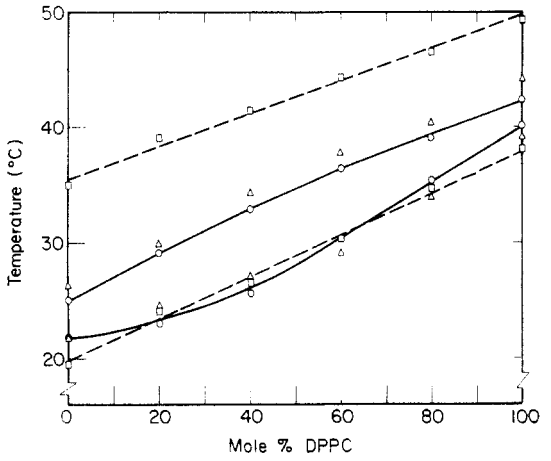

FIGURE 5: Phase diagram for multibilayer liposomes determined from the fluorescence polarization data of Figure 3 (○). The experimental points correspond to the temperatures marking the beginning and end of a transition. Phase diagram for the micellar complexes (□); the data were obtained from Figure 4. Temperatures where apo A-I no longer reacts with PC samples to form micellar complexes, obtained by extrapolation of the results in Figure 2 to 0% yields (△).

in Figure 5 by indicating the temperature for the onset and end of the transitions. In addition, the results shown in Figure 2 were extrapolated to 0% yield in order to obtain approximate temperature ranges in which reactions with apolipoproteins can take place. These temperature regions, as seen in Figure 5, delimit fairly closely the regions where gel and liquid-crystalline phases coexist in PC liposomes.

Discussion

Apolipoproteins A-I from human and bovine sources interact with DMPC and with DPPC to form very similar micellar complexes. However, there are major differences in the rates of the reactions with both phosphatidylcholines, as noted by other investigators (Pownall et al., 1978; Swaney, 1980a,b). Whereas the DMPC reaction, at the T_m of the lipid, reaches equilibrium in 3 h, the estimated equilibration time for the DPPC interaction is of the order of days.

Assuming that the difference in the reaction rates is due to the difference in the stability of the DPPC bilayer over the DMPC bilayer, one can calculate the relative rates from the transition-state theory (Frost & Pearson, 1961). In this calculation, the free-energy difference between DPPC and DMPC bilayers is equated with the difference in activation energy and is attributed to the contributions of two additional methylene groups in DPPC to the hydrophobic interactions. According to Tanford (1973), each methylene group contributes −0.8 kcal/mol to the micellization process in the absence of strong electrostatic repulsions. The calculated ratio of DMPC/DPPC reaction rates is 14.1 compared to the experimentally determined ratio of 18.9 (Table I). Since these ratios are comparable, the rate-limiting step for the reaction of apo A-I with these PC bilayers to form micellar complexes very likely involves the destabilization of the lipid bilayers.

In terms of complex properties, the DMPC and DPPC derivatives are similar in size (around 2×10^5 molecular weights) but somewhat different in stoichiometry (Table III). Electron micrographs of negatively stained preparations, using phosphotungstic acid, show discoidal complexes of similar dimensions with both lipids (Wetterau and A. Jonas, unpublished experiments). Swaney (1980b) points out that the complexes of DMPC enriched with DPPC or distearoylphosphatidylcholine are thicker than complexes of DMPC alone, as expected from their bilayer–disk structures.

Since in apo A-I·DMPC complexes, the apolipoprotein, in α-helical conformation, covers the acyl chains of the PC cylinder in a protein annulus (Tall et al., 1977; Wlodawer et al., 1979; Atkinson et al., 1980), one can calculate and compare the side areas of DMPC and DPPC cylinders corresponding to the observed complex stoichiometries. When the length of two fatty acyl chains is 36.3 and 41.4 Å for DMPC and DPPC, respectively, and the area per PC molecule in the gel state is 41 Å^2 (Tardieu et al., 1973), the calculated cylinder side areas for the DMPC and DPPC complexes are 9700 and 9400 Å^2, respectively. The projected area of an apo A-I monomer, assumed to be 100% α helical and lying flat on a surface, would be approximately 4600 Å^2. Thus, the DMPC and DPPC complexes could accommodate two apo A-I molecules each. The size of the complexes appears to be defined by the apolipoprotein content.

The temperature dependence of DMPC liposome interactions with apo A-I was first examined by Pownall et al. (1978). They showed that the optimal reaction rates occurred at the T_m of the lipid. The presence of lattice defects at the T_m was suggested to facilitate protein penetration and eventual disruption of the bilayer. In Figure 2, we show that multibilayer liposomes of DPPC and mixtures of DPPC and DMPC react most rapidly at the T_m of the lipid sample. By extrapolation to 0% yields, we estimate a temperature range where the formation of micellar complexes can occur for each mixture. When plotted on Figure 5, these data indicate that for liposomes the reaction is essentially limited to the region of the phase diagram where gel and liquid-cryatalline phases coexist. Since the lipid composition of the isolated complexes is identical with that of the starting mixtures, it is evident that equilibration between gel and liquid-crystalline phases at T_m must be much more rapid than the formation of the apolipoprotein–lipid complex. For distearoylphosphatidylcholine and DMPC mixtures, which are not ideally miscible as the DPPC and DMPC mixtures examined here, Swaney (1980a) indicates that reaction of apo A-I with the mixtures coincides with a portion of the solidus line of the corresponding phase diagram. In contrast, we observe optimal reaction at the midpoint of the transition and essentially no reaction at the onset and end of the transitions, i.e., at the solidus or liquidus curves of the DPPC–DMPC phase diagram. The interaction in our system appears to depend only on the average frequency of lattice defects.

Figures 4 and 5 describe the phase transition behavior of isolated complexes. The effect of the protein on the bilayers containing from 200 to 280 PC monomers is to restrict their mobility above the T_m of the complex and to increase it slightly below T_m, relative to the corresponding liposome behavior. The increase in the T_m of 3–4 °C from liposome to complex can be attributed to the stabilizing effect of the protein on the gel state of the complex lipid and the broadening of the transition to the decrease in the cooperative unit size and to the heterogeneity of the lipid environments in the complexes.

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Mechanism of Cholesterol Exchange between Phospholipid Vesicles†

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ABSTRACT: The kinetics of cholesterol exchange between two populations of small unilamellar vesicles has been investigated. There is no change in the initial rate of this exchange process over a 100-fold change in the acceptor vesicle concentration at a constant donor concentration. These results are not consistent with a collision-dependent exchange mechanism. In support of transfer via the aqueous phase, the inclusion of a negatively charged lipid into the vesicles did not affect the exchange rate. Evidence for a water-soluble pool of cholesterol that had partitioned out of the vesicle was obtained. Finally, cholesterol exchange was observed when donor and acceptor membranes were separated by a barrier through which neither could pass. These data together support our contention that the exchange of cholesterol between these vesicles involves a water-soluble intermediate.

The spontaneous exchange of cholesterol between plasma and red blood cells was first reported by Hagerman & Gould (1951). Since then it has been demonstrated to occur in a variety of biological membranes, lipoproteins, and liposomes (Bruckdorfer & Graham, 1976; Bell, 1976). Although cholesterol exchange is well documented, the mechanism of this process is not well understood. Two possibilities have been proposed. The first, originally suggested by Hagerman & Gould (1951), involves the partitioning of cholesterol out of the membrane into the aqueous phase which then acts as an intermediate in the equilibration process. A second mechanism first outlined by Gurd (1960) suggests that cholesterol exchange between membranes takes place upon contact of the membranes as a result of collision. This latter proposal, which also applied to exchange of phospholipids, was based solely on the view that cholesterol and phospholipids are essentially insoluble in water.

Evidence in favor of the collision-dependent mechanism for cholesterol exchange has been presented in several studies (Bruckdorfer & Green, 1967; Lenard & Rothman, 1976; Haran & Shporer, 1977; Poznansky & Lange, 1978; Moore et al., 1978; Patzer et al., 1978; Jonas & Maine, 1979; Giraud & Claret, 1979; Gottlieb, 1980), although in some of these the data on which the conclusions were based is somewhat scant. In addition, Bruckdorfer & Graham (1976) argued that the involvement of an aqueous cholesterol intermediate could be dismissed, because Gould et al. (1955) had reported that they were unable to observe cholesterol exchange between membranes that were separated from each other by a dialysis membrane. The reference quoted by Bruckdorfer & Graham (1976) does not contain this information. Furthermore, in a search of Gould's publications we have been unable to find the source of the data cited.

In marked contrast to the above-mentioned studies are those in which the spontaneous exchange of phospholipids between small unilamellar vesicles (SUV's)[1] was examined (Martin & MacDonald, 1976; Duckwitz-Peterlein et al., 1977; Roseman & Thompson, 1980). These authors showed that the rate of this exchange process is independent of the acceptor membrane concentration, from which it was concluded that the spontaneous exchange of phospholipids between SUV's occurs via the aqueous phase and does not involve membrane collisions. Similar conclusions have been reached for the transfer of pyrene (Charlton et al., 1976), a fluorescent cholesterol analogue (Kao et al., 1977), and a diglyceride (Charlton et al., 1978) between high-density lipoproteins. Furthermore, evi-

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[1] Abbreviations used: SUV, small unilamellar vesicle; MLV, multilamellar vesicle; EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane.

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