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Cholesterol Desorption from Clusters of Phosphatidylcholine and Cholesterol in Unilamellar Vesicle Bilayers during Lipid Transfer or Exchange†

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ABSTRACT: The rate of [4-^{14}C]cholesterol transfer from phosphatidylcholine (PC) small unilamellar vesicles of different acyl chain compositions and cholesterol concentrations has been followed as a function of temperature in the presence of excess acceptor vesicles. Cholesterol–PC vesicles containing either egg PC, dimyristoyl-PC (DMPC), or dipalmitoyl-PC (DPPC) were used as either donor or acceptor vesicles. One population of vesicles contained 15 mol % dicetyl phosphate to confer a negative charge so that the donor and acceptor vesicles could be separated rapidly by ion-exchange chromatography. The rate of cholesterol transfer from negatively charged 20 mol % cholesterol–egg yolk PC vesicles to pure egg PC, DMPC, or DPPC vesicles was followed over a temperature range which included the gel to liquid-crystal transition temperatures (T_c) of the saturated PC acceptor vesicles. The Arrhenius plots for all three systems are linear and superimposable so that the activation energy (E_a) is close to 88 kJ/mol in every case. Thus, neither the rate nor the E_a for transfer is dependent upon the physical state of the hydrocarbon chains in the acceptor vesicles when acceptor vesicles are in excess; this is consistent with earlier findings that under such conditions the rate of cholesterol transfer is determined by the rate of cholesterol desorption from the donor vesicle bilayer into the aqueous phase. The rate of cholesterol desorption from donor vesicles containing 1 mol % cholesterol in either egg PC, DMPC, or DPPC bilayers was followed at temperatures above and below the T_c of the donor vesicles. The rate constant for cholesterol desorption from egg PC bilayers is greater by an order of magnitude than for DMPC or DPPC bilayers. For all three host lipids, the Arrhenius plots do not show any significant deviations from linearity, and the E_a values above and below the T_c of the DMPC or DPPC vesicles do not differ significantly. Thus, the rate of cholesterol desorption is not influenced by the physical state of the phospholipids which can undergo chain melting in the donor bilayer but is strongly dependent on nearest-neighbor interactions. The first-order rate constant for cholesterol desorption from egg PC bilayers containing 5–40 mol % cholesterol is essentially constant ranging from 0.81×10^{-4} s^{-1} to 1.33×10^{-4} s^{-1}. These data suggest that cholesterol desorbs from a region of the bilayer which is laterally phase separated from the free-melting phospholipid. The cholesterol flux data are analyzed in terms of a model in which cholesterol desorption occurs from equimolar cholesterol–PC clusters in the bilayer. The rate of cholesterol desorption from PC bilayers containing 1–40 mol % cholesterol is proportional to the fraction of bilayer covered by equimolar PC–cholesterol clusters and is sensitive to the cholesterol–PC hydrocarbon chain interactions in such clusters.

An important aspect of cellular lipid metabolism is the exchange and transfer of cholesterol between cell membranes and lipoproteins [for a review, see Smith & Scow (1979)]. In a previous report (Phillips et al., 1980) we have shown that transfer of cholesterol from cells in culture to acceptors in the medium proceeds by diffusion of cholesterol through the aqueous phase. In order to investigate lipid exchange under carefully controlled conditions, we have also followed cholesterol and phosphatidylcholine (PC)[1] exchange between unilamellar vesicles (McLean & Phillips, 1981). In this system aqueous diffusion is also operative for exchange of both bilayer lipids. This conclusion for cholesterol exchange between unilamellar vesicles has been confirmed by Backer & Dawidowicz (1981). Aqueous diffusion proceeds as a two-step process: (1) desorption of cholesterol from a donor bilayer membrane into the aqueous phase and (2) adsorption of cholesterol from the aqueous phase into an acceptor bilayer. When acceptor vesicles are in excess, desorption is the rate-limiting step for exchange, and the rate of cholesterol exchange is independent of the concentration of the acceptor vesicles in the incubation mixture (McLean & Phillips, 1981).

A number of workers (Bloj & Zilversmit, 1977; Poznansky & Lange, 1978; Nakagawa et al., 1979) have shown that the rate of cholesterol exchange from sonicated cholesterol–PC donor vesicles of different acyl chain composition to various acceptors depends on the acyl chain composition of the donor vesicle bilayer PC. These data suggest that the physical state

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[1] Abbreviations: DEAE, diethylaminoethyl; PC, phosphatidylcholine; DMPC, dimyristoylphosphatidylcholine; DPPC, dipalmitoylphosphatidylcholine; SEM, standard error of the mean; PNA, 9-(3-pyrenyl)nonanoic acid; DSC, differential scanning calorimetry.

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of the donor bilayer phospholipids may play a role in determining the overall rate of cholesterol exchange. However, since the physical state of the acceptors was not kept constant during these studies, whether or not the composition and physical state of the donor vesicle are important in determining the rate of the cholesterol desorption step remains an open question. A particular omission is the lack of a systematic investigation of the influence of the cholesterol:PC mole ratio of the donor bilayer. The physical state of cholesterol in PC bilayers has been the subject of active research, much of which has focused on the well-known condensing effect of cholesterol on bilayer phospholipids [for reviews, see Phillips (1972) and Demel & DeKruyff (1976)]. Recently, evidence has accumulated that lateral phase separation can occur with cholesterol–phosphatidylcholine clusters separating from pure PC in mixed bilayers. The first direct evidence for such a phase separation of cholesterol and PC was based on the NMR spectrum of cholesterol–PC bilayers (Darke et al., 1971, 1972). Various stoichiometries have been proposed for the clusters of cholesterol and PC [1:1 mol/mol cholesterol:PC, Phillips & Finer (1974); 1:2, Hinz & Sturtevant (1972), Engelman & Rothman (1972), and Gershfeld (1978); 1:4, Rubenstein et al. (1980), Copeland & McConnell (1980), and Melchior et al. (1980)]. Rand et al. (1980) have also proposed a 1:8 cluster at cholesterol concentratioins between 3 mol % and 10 mol %.

Providing that phase separation between free PC and PC–cholesterol complexes occurs, three predictions concerning the rate of cholesterol desorption from bilayers may be made. (1) The rate of desorption of cholesterol out of the cluster should not be influenced by the physical state of the PC outside the cluster. (2) Over a region where the stoichiometry of the cluster is constant, the frequency of desorption per cholesterol molecule should be independent of the overall concentration of cholesterol in the bilayer. (3) The rate of cholesterol desorption should be a function of the stoichiometry of the cluster. In this study, the above predictions are tested by following the rate of cholesterol desorption out of unilamellar vesicles of various compositions. The data for the rate of cholesterol desorption are consistent with a model in which cholesterol desorbs from that fraction of the bilayer surface which is covered by an equimolar cluster of cholesterol and PC.

Experimental Procedures

Materials. The purity and source of all lipids, [7-^{3}H]-cholesteryl oleate and [4-^{14}C]cholesterol have been described previously (McLean & Phillips, 1981). Before incubation [4-^{14}C]cholesterol was >98% pure by thin-layer chromatography. After incubation of [4-^{14}C]cholesterol-containing unilamellar vesicles for 2 h at 37 °C, >97% of the labeled cholesterol cochromatographed with a cholesterol standard.

Preparation of Cholesterol-Rich Dispersions. Cholesterol-rich PC dispersions were prepared as described by Collins & Phillips (1982). Cholesterol and dipalmitoyl-PC with butylated hydroxytoluene as antioxidant (0.01% w/w of PC) were mixed in chloroform and dried under N_2 to a thin film on the walls of a test tube. Traces of remaining solvent were removed by drying for 2 h at 40 °C under vacuum. The dry lipid mixture was taken up in 7 mL of 20 mM sodium phosphate buffer, pH 6.0, and immersed in a 50 °C water bath. The dispersions were sonicated under N_2 for 60 min (in 10-min periods separated by 2-min cooling periods) on setting 4 by using a Branson Model 350 sonifier equipped with a tapered microtip immersed 2 cm into the dispersions. After sonication the dispersions were centrifuged at 4 °C for 30 min at 20 000 rpm ($37000g_{max}$) in a Beckman type 40 rotor to remove fragments of titanium and undispersed lipid. Following filtration through a 0.4-μm Nuclepore polycarbonate membrane, the dispersions were stored under N_2 and used within 2 days of preparation.

Separation of Vesicles. Unilamellar vesicles were prepared in 20 mM sodium phosphate buffer, pH 6.0, by a modification of the procedure of Barenholz et al. (1977) as described previously (McLean & Phillips, 1981). Two populations of vesicles, one of which contained 15 mol % dicetyl phosphate to confer a negative charge and the other of which was neutral, were rapidly separated on short columns of DEAE-Sepharose CL-6B as described previously (McLean & Phillips, 1981) by a modification of the procedure of Hellings et al. (1974) and van den Besselaar et al. (1975). The donor vesicles (either neutral or negatively charged) were prepared with [4-^{14}C]-cholesterol as the exchangeable lipid; the neutral vesicles contained [7-^{3}H]cholesteryl oleate to monitor recovery of the eluted vesicles. Typically, <1% of the charged vesicles and 80–90% of the neutral vesicles were recovered in the eluate. After incubation of the vesicles for 12 h at 37 °C, >90% of the vesicles remained unilamellar with a modal diameter of 23 ± 2 nm as judged by negative-stain electron microscopy, and <5% of the charged or neutral vesicles appeared in the void volume of a Sepharose CL-2B column with no significant shift in the K_{av} of the unilamellar vesicles (McLean & Phillips, 1981).

Kinetic Analysis. Kinetic data were analyzed according to isotope exchange kinetics (McKay, 1938). For the case where the donor vesicles are negatively charged, the fraction of label transferred at time t is given by

$$X = \frac{(^{14}C/^{3}H)_t - (^{14}C/^{3}H)_0}{(^{14}C/^{3}H)_{mix}}$$

Here $(^{14}C/^{3}H)_t$, $(^{14}C/^{3}H)_0$, and $(^{14}C/^{3}H)_{mix}$ represent the ratio of ^{14}C to ^{3}H in the eluate at time t, at time 0, and in the incubation mixture, respectively. For neutral donor vesicles

$$X = 1 - \frac{(^{14}C/^{3}H)_t}{(^{14}C/^{3}H)_{mix}}$$

Where the concentration of acceptor particles is a and donor particles is b, the rate of lipid transfer from donor to acceptor particles is given by the linear expression

$$kt = -\left(\frac{a}{a+b}\right) \ln\left(1 - \frac{X}{X_\infty}\right) \qquad (1)$$

The value of k was determined by the best fit of eq 1 to the experimental data using a least-squares procedure. An equal distribution of labeled lipid between donor and acceptor particles at infinite time was assumed so that $X_\infty = a/(a + b)$. This analysis corrects for back-exchange of label so that the unidirectional rate of transfer of lipid from donor to acceptor vesicles is followed.

Differential Scanning Calorimetry. Vesicles of cholesterol and PC were prepared at a concentration of 100 mg/mL as described above but without centrifugation. A 50-μL aliquot of the vesicles was sealed in a stainless steel sample pan and placed in a Perkin-Elmer DSC-2 calorimeter. Heating scans over the temperature range 12–50 °C were performed at a heating rate of 0.5 or 1.0 deg min^{-1} against a reference pan containing 50 μL of distilled water. The calorimeter was calibrated, and the data were analyzed, as described previously (Collins & Phillips, 1982).

Analytical Procedures. The procedures for electron microscopy and gel filtration have been described elsewhere (McLean & Phillips, 1981). Phospholipid concentrations were

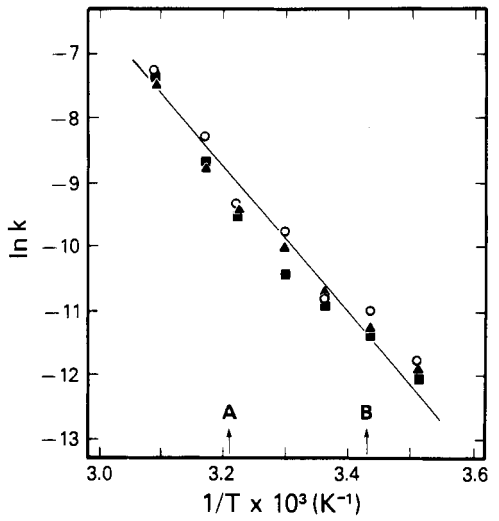

FIGURE 1: Cholesterol transfer to pure phosphatidylcholine vesicles of differing acyl chain compositions. The transfer of [4-^{14}C]cholesterol from 20 mol % cholesterol, 15 mol % dicetyl phosphate, and 65 mol % egg yolk phosphatidylcholine vesicles to pure phosphatidylcholine vesicles of differing acyl chain compositions was followed as described under Experimental Procedures. Each point represents the average of two to four experiments. Each experimental rate constant (s^{-1}) was determined from four aliquots taken at six time points. The range of rate constants is ±5–10% of the average value. Donor vesicles contained [4-^{14}C]cholesterol as the exchangeable lipid; neutral acceptor vesicles contained [7-^{3}H]cholesteryl oleate to monitor recovery. In all experiments the ratio of acceptors:donors was at least 1:1. Acceptor vesicles comprised egg yolk PC (○), DMPC (■), or DPPC (▲). The solid line represents the best fit to all of the data by linear regression. The gel to liquid-crystal transition temperatures of DPPC and DMPC unilamellar vesicles are shown by arrows A and B, respectively.

estimated by analysis for phosphorus by the method of Sokoloff & Rothblat (1974). Cholesterol was analyzed with the Liebermann and Burchard reagent as described by Billimoria & James (1960).

Results

Effect of Acceptor Bilayer Physical State on the Rate of Cholesterol Transfer. Figure 1 gives the rate of cholesterol transfer from negatively charged 20 mol % cholesterol–egg yolk PC unilamellar vesicles to pure PC vesicles of different acyl chain compositions. The rate of transfer was followed over a range of temperatures which includes the phase transition temperatures of the saturated PC acceptor bilayers [23 °C for dimyristoyl-PC (DMPC) and 41 °C for dipalmitoyl-PC (DPPC); Chapman et al., 1967; Phillips et al., 1969]. An analysis of variance of the activation energy data (Sokal & Rohlf, 1969) shows that no significant deviations from linearity (at $p < 0.05$) are present when the acceptor vesicles are egg yolk PC, DMPC, or DPPC. In fact, all three Arrhenius plots are superimposable: there is no significant difference (at $p < 0.05$) among the slopes or intercepts of the three lines. The activation energy for egg yolk PC acceptors is 88.5 ± 6.7 kJ mol^{-1} (n = 18), for DMPC acceptor vesicles is 90.4 ± 7.5 kJ mol^{-1} (n = 20), and for DPPC acceptor vesicles is 87.3 ± 7.1 kJ mol^{-1} (n = 19). As expected for aqueous diffusion of cholesterol, where the rate-limiting step for exchange is desorption, neither the rate nor the activation energy for transfer is altered by substituting saturated PC acyl chains for egg yolk PC in the acceptor vesicles when the acceptor vesicles are in excess.

Effect of Donor Vesicle Physical State on the Rate of Cholesterol Exchange. When acceptor vesicles are present in excess, the rate of cholesterol exchange is determined by the rate of cholesterol desorption from the donor bilayer into the aqueous phase (McLean & Phillips, 1981) and is independent of the physical state of the acceptor vesicles (Figure 1). The rate of cholesterol desorption from bilayers comprising different PC acyl chains was followed at temperatures above and below the phase transition temperatures of the donor vesicles to test whether the donor vesicle lipid influences the rate of cholesterol desorption out of a PC bilayer. A minimal amount of cholesterol was incorporated into the donor bilayer vesicle in order to obtain a sharp PC phase transition. In these experiments, a saturated PC bilayer with 1 mol % cholesterol was used as the donor vesicle.

The size by negative-stain electron microscopy of vesicles comprising 1 mol % cholesterol–DMPC is similar to that of cholesterol–egg yolk PC bilayer vesicles (cf. 21 ± 4 and 23 ± 2 nm; McLean & Phillips, 1981). It should be noted that the radius of the 1 mol % cholesterol–DMPC vesicles is similar at 4 and 37 °C by negative-stain electron microscopy (cf. Watts et al., 1978; Cornell et al., 1981). The size distribution of 1 mol % cholesterol–DMPC bilayer vesicles on Sepharose CL-2B columns is also similar to that of egg yolk PC vesicles at 37 °C, but a small amount of fusion is evident on the columns when run at 4 °C. This may be an artifact of the gel filtration procedure since the electron microscopy results do not agree (cf. Watts et al., 1978). The size of 1 mol % cholesterol–DPPC vesicles is also similar to that of cholesterol–egg yolk PC vesicles, although these vesicles collapse when dried on an electron microscope grid and stained below their phase transition temperature. This results in flattened disks of 24–31-nm diameter which stack in clusters. Disks of this size correspond to collapsed spheres of 17–22 nm, in good agreement with the 23 ± 2 nm diameter of 20 mol % cholesterol–egg yolk PC vesicles measured previously by negative-stain electron microscopy (McLean & Phillips, 1981).

Both 1 mol % cholesterol–DMPC and 1 mol % cholesterol–DPPC vesicles exhibit a broad endothermic transition on heating and a similar exothermic transition on cooling which corresponds to the gel to liquid-crystal phase transition of the respective phosphatidylcholines in the bilayer vesicles. For 1 mol % cholesterol–DMPC vesicles, two phase transitions are evident in DSC heating runs: one is centered at 18.7 °C and the second at 24.5 °C, while for 1 mol % cholesterol–DPPC vesicles, the corresponding phase transition temperatures are 38.6 and 42 °C. The phase transition temperatures of both vesicle preparations correspond well with literature values for unilamellar DPPC vesicles (Suurkusk et al., 1976; Papahadjopoulos et al., 1974, 1976) and for hand-shaken DMPC liposomes (Phillips et al., 1969).

Figure 2 gives the rate of cholesterol exchange between vesicles comprising 1 mol % cholesterol in PC bilayers of various acyl chain compositions. In these experiments, the acceptor vesicles were in excess so that desorption was the rate-limiting step for exchange. Under these conditions, only the physical state of the donor vesicles influences the overall rate of cholesterol exchange (Figure 1), and the more rapid rate of cholesterol desorption from vesicles comprising cholesterol and egg PC is not the result of a slower penetration of cholesterol into the DMPC or DPPC acceptor bilayer. The rate data fall into two groups which differ significantly (at $p < 0.05$), corresponding to PC vesicles comprising one or two saturated acyl chains.

In Figure 2, the rate of exchange follows the Arrhenius rate expression over a range of temperatures which includes the phase transition temperatures of both of the saturated PC vesicles. An analysis of variance of the linear regression data (Sokal & Rohlf, 1969) shows that there are no significant

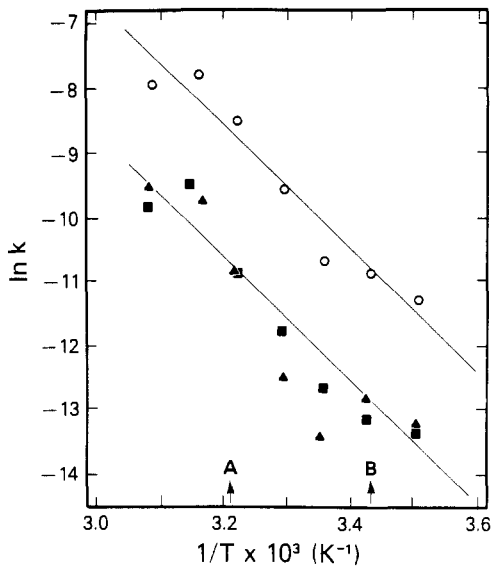

FIGURE 2: Cholesterol exchange from 1 mol % cholesterol–phosphatidylcholine donor vesicles of differing acyl chain compositions. The rate of [4-^{14}C]cholesterol transfer from 1 mol % cholesterol and 99 mol % phosphatidylcholine vesicles of differing acyl chain compositions to negatively charged vesicles of similar composition was followed over a range of temperatures as described under Experimental Procedures and in Figure 1. The host lipid comprised egg yolk PC (○), DMPC, (■), or DPPC (▲). The lines are the best fit to the two groups of data by linear regression. The gel to liquid-crystal transition temperatures of DPPC and DMPC unilamellar vesicles are shown by arrows A and B, respectively.

deviations from linearity in any of the Arrhenius plots of Figure 2 ($p < 0.05$) and that the activation energies above and below the phase transition temperatures of the 1 mol % cholesterol–DMPC or 1 mol % cholesterol–DPPC vesicles do not differ significantly (at $p < 0.05$). For each of the three donor vesicle preparations the activation energies are not significantly different ($p < 0.05$). The activation energy for cholesterol desorption from 1 mol % cholesterol–egg yolk PC donor vesicles is 80.3 ± 9.3 kJ mol^{-1} ($n = 14$), for 1 mol % cholesterol–DMPC donors is 78.9 ± 8.9 kJ mol^{-1} ($n = 17$), and for 1 mol % cholesterol–DPPC donors is 86.3 ± 13.7 kJ mol^{-1} ($n = 12$). It is important to note that 1 mol % cholesterol–egg yolk PC vesicles do not undergo a phase transition over the temperatures included in this study [the phase transition temperature of egg yolk PC is between −15 °C and −7 °C (Szoka & Papahadjopoulos, 1980)]. The absence of a break in the Arrhenius plot for cholesterol exchange is in contrast to the results of Doody et al. (1980) for 9-(3-pyrenyl)nonanoic acid (PNA) transfer between dimyristoyl-PC bilayers.

Rate Constants for Cholesterol Exchange as a Function of the Cholesterol Concentration of the Donor Bilayer. The rate constant for cholesterol exchange at different mole fractions of cholesterol in donor bilayers containing egg yolk PC is given in Table I. Under the experimental conditions of this table (excess acceptors), the rate-limiting step in cholesterol exchange is desorption of cholesterol from the donor bilayer into the aqueous phase. The rate constants for cholesterol desorption decrease significantly (at $p < 0.05$) with increasing cholesterol concentration in a nonlinear fashion. This decrease in the rate of cholesterol desorption is much less marked than the order of magnitude decrease observed when the host egg PC is replaced by a fully saturated PC [cf. Figure 2 and Nakagawa et al. (1979)]. In contrast, Poznansky & Czekanski (1979) found an increase in the rate of cholesterol exchange by a factor of approximately 1.4 in dioleoyl-PC bilayers when the concentration of cholesterol was increased from 19 to 48 mol %. These authors also observed a sharp decrease in the activation energy of exchange when the mole percent of cholesterol increased from 20 to 30. Inspection of Table I indicates that this is not the case for cholesterol–egg yolk PC vesicles. There is no significant difference ($p < 0.05$) among the four activation energies at different cholesterol concentrations in the donor vesicle bilayer.

Table I: Cholesterol Exchange Rate Constants as a Function of Cholesterol Concentration[a]

mol % cholesterol	neutral donors k (×10^{-4} s^{-1})	charged donors k (×10^{-4} s^{-1})	E_a (kJ mol^{-1})
1	2.07 ± 0.31 (3)		80.3 ± 9.3 (14)
5	1.17 ± 0.01 (3)		
10	1.33 ± 0.29 (3)	1.22 (1)	74.2 ± 2.7 (14)
20	0.99 ± 0.16 (3)	0.89 ± 0.11 (5)[b]	73.0 ± 5.5 (44)[b]
40	0.81 ± 0.07 (2)	0.88 (1)[c]	67.9 ± 5.7 (6)[c]

[a] The rate of [4-^{14}C]cholesterol transfer from neutral or charged vesicle donors containing the indicated concentrations of cholesterol to charged or neutral vesicles of similar composition was followed over a range of temperatures as described under Experimental Procedures. The half-times for exchange are at 37 °C. [b] McLean & Phillips (1981). [c] Phillips et al. (1980). k and E_a data are ±SEM.

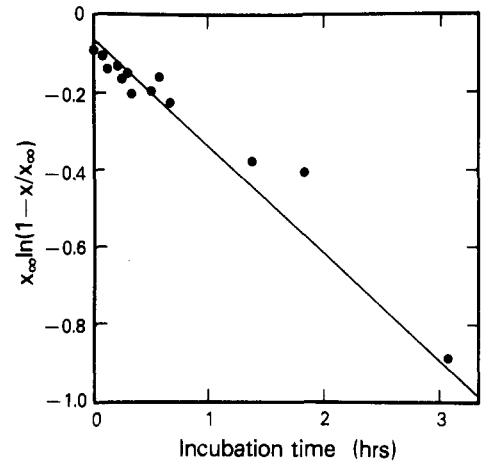

FIGURE 3: Isotope-exchange plot of the transfer of [4-^{14}C]cholesterol out of cholesterol-rich dispersions. Cholesterol-rich dispersions were prepared with [4-^{14}C]cholesterol to a molar ratio of 3.4:1 cholesterol:dipalmitoylphosphatidylcholine as described under Experimental Procedures. Dispersions were incubated with 15 mol % dicetyl phosphate–egg yolk phosphatidylcholine vesicles and separated at intervals as described under Experimental Procedures.

Cholesterol Transfer Out of Cholesterol-Rich Dispersions. Dispersions containing 50 mol % cholesterol comprise multilamellar and unilamellar vesicles with diameters from 20 to 180 nm and throw off cholesterol monohydrate crystals on storage (Collins & Phillips, 1982). These vesicles may be used to obtain some information on the rate of cholesterol desorption from bilayers whose cholesterol:PC molar ratio is greater than 1:1. Figure 3 gives an isotope-exchange plot of cholesterol transfer from cholesterol–DPPC dispersions of molar ratio 3.4:1 (cholesterol:PC) to charged egg yolk PC bilayer vesicles at 37 °C. A rapid initial phase is evident which cannot be measured by using the ion-exchange column separation technique and which accounts for 9% of the cholesterol transferred. This rapid initial phase of cholesterol desorption from cholesterol-rich dispersions is not due to oxidation of the cholesterol during sonication. The thin-layer chromatography results described under Materials indicate that less than 3% of the [4-^{14}C]cholesterol is oxidized under the conditions of sonication used in these experiments (which include butylated

hydroxytoluene as an antioxidant). At least an additional 50% of the total pool of cholesterol transfers according to first-order kinetics with a half-time of 2.9 h in two experiments. This half-time may be compared to that for cholesterol exchange between 1 mol % cholesterol–dipalmitoyl-PC bilayers (10.3 h at 37 °C).

Discussion

In this report, the rate of [4-^{14}C]cholesterol transfer from PC bilayer vesicles of different acyl chain compositions and cholesterol concentrations has been followed as a function of temperature in the presence of excess acceptor vesicles. Under these conditions, the rate of cholesterol transfer is determined by the rate of cholesterol desorption from the donor vesicle bilayer into the aqueous phase (McLean & Phillips, 1981). Since desorption is the rate-limiting step for exchange, the data of Figure 1 are consistent with the aqueous diffusion model of lipid exchange. It is important to note that the rate of desorption is independent of the physical state of the acceptor bilayer lipid when the acceptor vesicles are present in excess. Under these conditions, the effect of the physical state of the donor bilayer lipid on the rate of cholesterol exchange can be separated from the effect of the acceptor bilayer. This method provides the first system in which cholesterol desorption from the donor bilayer vesicle into the aqueous phase may be examined in terms of the composition of the donor bilayer lipid and the phase behavior of cholesterol–phospholipid dispersions.

Cholesterol–Phospholipid Interactions and the Rate of Cholesterol Desorption. The interactions of cholesterol molecules in a bilayer membrane are dependent on the nature of the nearest-neighbor molecules. As far as the host phospholipid is concerned, the interactions are a function of the fatty acid composition (Phillips, 1972). Consequently, cholesterol molecules exchange more rapidly from unsaturated PC bilayers. The data in Figure 2 comparing k for cholesterol desorption for vesicles of egg PC, DMPC, and DPPC are consistent with three previous reports (Bloj & Zilversmit, 1977; Poznansky & Lange, 1978; Nakagawa et al., 1979) showing that $t_{1/2}$ for cholesterol desorption from egg PC < DMPC ≈ DPPC.

The fact that k remains essentially constant as the cholesterol content is increased from 5 to 40 mol % (Table I) suggests that the local cholesterol interactions remain unchanged in this region and apparently only alter significantly when the cholesterol:PC ratio is raised above 1:1. The predominant structure in cholesterol–DPPC dispersions prepared at cholesterol concentrations greater than 50 mol % cholesterol is a bilayer of cholesterol and DPPC (Collins & Phillips, 1982). This lamellar structure prevails up to cholesterol:PC molar ratios of 4:1. Presumably, the nearest neighbors of cholesterol are predominantly cholesterol rather than PC in these cholesterol-rich dispersions. The rate of desorption from the bilayer evident after the initial rapid phase (Figure 3) is faster than that observed for cholesterol–DPPC bilayers of less than 50 mol % cholesterol; this is most likely due to the altered cholesterol–nearest-neighbor interactions in the bilayer. The rapid initial phase in Figure 3 may be due to loosely absorbed cholesterol molecules on the vesicle surface which are already partially hydrated.

Cholesterol Desorption from a Cluster of Cholesterol and Phosphatidylcholine. Two limiting models may describe the physical state of cholesterol in a bilayer. The first is that cholesterol is randomly distributed in the bilayer up to a saturation concentration. The second is that at low cholesterol concentrations, cholesterol separates with PC in a cluster of definite stoichiometry in equilibrium with free PC. In the case of cholesterol desorption out of a bilayer where cholesterol is randomly distributed, it is expected that the rate of desorption would be influenced by the physical state of the surrounding lipid environment. In contrast, if cholesterol desorbed from a region where a cluster of cholesterol and PC phase separated from the surrounding PC, the rate of cholesterol desorption would be independent of the physical state of the phospholipids outside the cluster. According to the DSC data presented in this paper, vesicles comprising 1 mol % cholesterol in saturated PC bilayers show a definite phase transition between the gel and liquid-crystalline states. This phase transition (Ladbrooke et al., 1968) corresponds to the melting behavior of the free phospholipid (Estep et al., 1978; Mabrey et al., 1978). It is clear from Figure 2 that the rate of cholesterol desorption is not influenced significantly by the physical state of the free-melting phospholipids but is influenced by the degree of unsaturation of the PC acyl chains. These data provide convincing evidence that cholesterol desorption proceeds from a region which is phase separated from the free-melting phospholipid. It is interesting to note that the rate of transfer of PNA from DMPC bilayers into the aqueous phase is a sensitive function of the phase of the bilayer lipids (Doody et al., 1980). In this case, the probe is probably randomly distributed in the bilayer and samples the free phospholipid environment in contrast to cholesterol which phase separates from free PC.

For cholesterol desorption out of a cluster of given stoichiometry, the flux of molecules out of the vesicle bilayer should be a linear function of the fraction of bilayer surface covered by such clusters. The flux of cholesterol out of the donor bilayer may be calculated directly up to 30 mol % cholesterol by using the size and molecular weight data of Newman & Huang (1975) for neutral cholesterol–egg yolk PC vesicles using $J = kc/A$, where J is the interfacial flux out of the donor bilayer (mol cm^{-2} s^{-1}), k is the rate constant for exchange, c is the moles of cholesterol per vesicle, and A is the surface area of a vesicle. The radius of vesicles comprising 40 mol % cholesterol cannot be calculated from Stokes' law since the hydrodynamic particles are no longer spherical above 32 mol % cholesterol (Newman & Huang, 1975). Molecular weights obtained by using the Svedberg equation are independent of the particle shape (Watts et al., 1978) and may be used in conjunction with measured hydrodynamic densities of the vesicles (Newman & Huang, 1975) to calculate the vesicle radius. The external radius, r_e, of 40 mol % cholesterol vesicles was calculated from the partial specific volume and molecular weight of the vesicles by using the expression

$$M = \frac{4}{3}\pi r_e^3 \left(\frac{N}{\rho_v}\right) - \frac{4}{3}\pi(r_e - d)^3 \left(\frac{N}{\rho_w}\right) \qquad (2)$$

Here M is the molecular weight of the vesicles, N is Avogadro's number, d is the vesicle bilayer thickness, and ρ_v and ρ_w are the densities of the vesicle and water, respectively. The X-ray diffraction data of Lecuyer & Dervichian (1969) give d = 2.94 nm for pure egg yolk PC vesicles and 3.26 nm for 40 mol % cholesterol–egg yolk PC vesicles. A molecular weight of 1.90 × 10^6 was obtained for egg yolk PC vesicles by using eq 2 and the measured hydrodynamic radius of the vesicle (Huang, 1969). This value is in good agreement with the hydrodynamic measurement obtained by application of the Svedberg equation (Huang, 1969). By use of a molecular weight obtained from the data of Newman & Huang (1975), the external radius of a 40 mol % cholesterol–egg yolk PC vesicle is 13.92 nm. This corresponds to an increase of 30% over the radius of a pure egg yolk PC bilayer. This increase is similar to that observed by gel filtration [50% (Gent & Prestegard, 1974)] and by

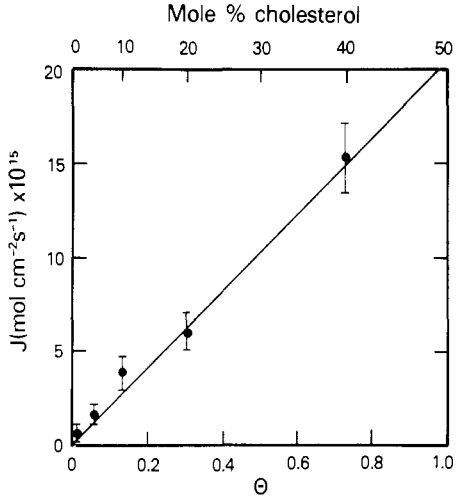

FIGURE 4: Flux of cholesterol out of cholesterol–egg yolk phosphatidylcholine vesicle bilayers as a function of the fraction of bilayer surface (θ) covered by an equimolar cluster of cholesterol and phosphatidylcholine. The transfer of [4-^{14}C]cholesterol from neutral vesicles of various mole fractions of cholesterol to charged vesicles of similar composition was followed as described under Experimental Procedures. The value of θ was calculated according to eq 3; the corresponding average values of the flux were calculated as described under Discussion. The values of J are plotted ±SEM.

freeze–etch electron microscopy [10% (Forge et al., 1978)].

In Figure 4 it has been presumed that the stoichiometry of the cluster is equimolar (cf. Phillips & Finer, 1974; Collins & Phillips, 1982), so that a cholesterol–egg PC bilayer will form two phases comprising a region of free PC and a region of cholesterol and PC in a 1:1 mole ratio. In excess water, the mean molecular area of a pure egg yolk PC bilayer is 63 Å^2 and an equimolar mixture of cholesterol and egg yolk PC has a mean molecular area of 43 Å^2 (Lecuyer & Dervichian, 1969). For a bilayer comprising X_c mole fraction of cholesterol, the fraction of surface θ which is covered by an equimolar cluster of PC and cholesterol is given by

$$\theta = \frac{2X_c(43)}{2X_c(43) + (1 - 2X_c)(63)} \quad (3)$$

where $1 - 2X_c$ is the mole fraction of PC outside the cluster. It is apparent from Figure 4 that the nonlinear relationship between the rate of cholesterol desorption and the mole fraction of cholesterol (Table I) is made linear when desorption from a cluster of PC and cholesterol on the surface of the vesicle is taken into account in the analysis of the kinetic data. Thus, desorption from the clusters occurs at a rate which is independent of the overall concentration of cholesterol in the bilayer. The linear relationship between the rate of cholesterol desorption from bilayers containing 1–40 mol % cholesterol and the fraction of bilayer covered by equimolar clusters of PC and cholesterol is consistent with a stoichiometry of the cluster of 1:1. The complete phase diagrams of the hydrated cholesterol–DPPC and cholesterol–DMPC systems support this conclusion (M. Forte and M. C. Phillips, unpublished results).

The above data are consistent with a model for cholesterol desorption in which the rate of cholesterol desorption, at mole fractions of cholesterol of less than 0.5, is primarily dictated by cholesterol–PC hydrocarbon chain interactions in an equimolar cholesterol–PC cluster in the bilayer. A number of workers (Hinz & Sturtevant, 1972; Engelman & Rothman, 1972; Gershfeld, 1978) have suggested that cholesterol phase separates with PC into a cluster of mole ratio approximately 1:2 cholesterol:PC in bulk dispersions of the two lipids. This behavior would result in the formation of a new phase above 33 mol % cholesterol. If clusters of two different stoichiometries were present at high cholesterol concentrations, presumably the rate of cholesterol desorption from the cholesterol-rich cluster would be faster than from the 1:2 clusters. This would result in a curvilinear dependence of the rate of cholesterol desorption on the fraction of bilayer covered by clusters of cholesterol and PC. For the data shown in Figure 4, no such curvilinear relationship is evident. Taken together with evidence for lateral phase separation in cholesterol–PC systems, these data suggest that cholesterol desorption proceeds from an equimolar cluster of cholesterol and PC which phase separates from bulk, free-melting phospholipid. Since the plasma membranes of mammalian cells comprise 30–40 mol % cholesterol, it is possible that cholesterol desorption from such membranes also proceeds from an equimolar cluster of cholesterol and phospholipid. The PC–cholesterol interactions in such clusters may contribute to variations in cholesterol efflux between different cell types (e.g., Phillips et al., 1980).

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Equilibrium Binding of Spin-Labeled Fatty Acids to Bovine Serum Albumin: Suitability as Surrogate Ligands for Natural Fatty Acids†

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ABSTRACT: Electron paramagnetic resonance (EPR) and saturation transfer EPR (ST-EPR) spectroscopies were used to characterize the binding of spin-labeled fatty acid (SLFA) to bovine serum albumin (BSA). Association constants of three stearic acid derivatives labeled with a nitroxyl radical at C-5, C-12, or C-16 were estimated by EPR spectroscopy as the ratio of SLFA to BSA was increased from about 0 to 9. The values were compared to those for unmodified stearate. With all three SLFA, it was apparent that the nitroxyl residue modified the binding pattern. For SLFA:BSA ratios up to 1, which probably involves the site(s) on BSA most specific for long-chain FA, the C-16 derivative bound with an affinity similar to that of the natural FA. At higher ratios, the association constants for this SLFA were lower than those for stearate. The C-12 and C-5 derivatives showed only low-affinity binding relative to stearate. The spectral parameter, W, was constant for SLFA:BSA ratios between 0 and 1 in the case of the C-16 compound, indicating physical homogeneity of the high-affinity binding site. At higher ratios, the spectra changed progressively, indicating inhomogeneity of the lower affinity binding sites although parallel changes in association constants were not observed. Changes in W due to Heisenberg spin exchange were ruled out. By examining the mobility profile of the bound SLFA by both EPR and ST-EPR techniques, it was shown that the nitroxyl group was maximally immobilized when attached near the center of the carbon chain of the bound SLFA.

Spin-labeled fatty acids (SLFA)[1] have been used by Morrisett et al. (1975) to characterize fatty acid binding to serum albumin by electron paramagnetic resonance (EPR) spectroscopy. This technique permits differentiation of free and bound SLFA, as illustrated in the study by Rehfield et al. (1978), and thus appears to simplify estimation of association constants for SLFA–serum albumin complexes. Rehfield et al. (1978) also used EPR to demonstrate a difference in binding of SLFA to human and bovine serum albumins. Furthermore, since the paramagnetic nitroxide group can be located at various positions along the backbone of the fatty acid molecule, determination of the site of maximal immobilization of the BSA-bound SLFA appeared possible. In such a study, Morrisett et al. (1975) concluded that the carboxyl terminus of stearic acid was more rigidly fixed than either the

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[1] Abbreviations: EPR, electron paramagnetic resonance; ST-EPR, saturation transfer electron paramagnetic resonance; SLFA, spin-labeled fatty acids; BSA, bovine serum albumin; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; I(1,14), 16-nitroxyl stearate; I(5,10), 12-nitroxyl stearate; I(12,3), 5-nitroxyl stearate (5-NS).

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