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Kinetics of Phosphatidylcholine and Lysophosphatidylcholine Exchange between Unilamellar Vesicles[†]

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ABSTRACT: The rates of exchange of phosphatidylcholine and lysophosphatidylcholine from unilamellar donor vesicles to acceptor vesicles of similar composition were followed in a protein-free system to establish the relationship between the rate of exchange and the aqueous-phase solubility of the lipid. Further, the rate of exchange of dimyristoylphosphatidylcholine (DMPC) between vesicles was examined over a range of temperatures to determine the effect of the lipid phase transition on the rate of lipid exchange. Intervesicular exchange of DMPC is faster than transbilayer exchange; lipid molecules in the outer monolayer of the bilayer exchange with $t_{1/2}$ = 2.0 h at 37 °C. A discontinuity is observed in Arrhenius plots of DMPC exchange; the activation energy over the temperature range 27–45 °C is 70 kJ mol^{-1}. The $t_{1/2}$ for DMPC exchange extrapolated to 24.5 °C (the phase transition temperature of the donor bilayer) is 6.5 h and from temperatures below 24 °C is 82.6 h. The differences in the thermodynamic parameters of activation for DMPC exchange above and below 24.5 °C are 25 kJ mol^{-1} for the activation enthalpy and 197 J mol^{-1} K^{-1} for the activation entropy. These differences are similar to the enthalpy and entropy changes associated with the gel to liquid-crystalline phase transition of DMPC. The rate of exchange of lysopalmitoylphosphatidylcholine (LPPC) was difficult to measure since LPPC transfers rapidly to the columns used for separating donor and acceptor vesicles; the $t_{1/2}$ for transfer is <2 min. LPPC at 5 mol % in cholesterol–egg PC vesicles does not affect the rate of cholesterol exchange. The rates of exchange of cholesterol, LPPC, DMPC, dipalmitoylphosphatidylcholine, and 1-palmitoyl-2-oleoylphosphatidylcholine were used to calculate activation free energies for exchange of each molecule. The activation free energies and free energies of transfer from self-micelles to water increase by 2.2 and 2.1 kJ mol^{-1} per methylene group, respectively. Thus, the free energy of transfer is a good predictor of the relative exchange rates of lipid molecules. However, the activation free energies are 30 ± 1 kJ mol^{-1} greater than the free energies of transfer. This excess free energy is proposed to be associated with restriction of the lipid molecule to the surface of the vesicle in the transition-state complex.

Lipid transfers between biological membranes, plasma lipoproteins, and bile acid micelles play an important role in lipid metabolism. Recently, a number of laboratories have shown that cholesterol and phosphatidylcholine exchange between unilamellar vesicles [Martin & MacDonald, 1976; Duckwitz-Peterlein et al., 1977; Thilo, 1977; Duckwitz-Peterlein & Moral, 1978; McLean & Phillips, 1981, 1982; Backer & Dawidowicz, 1981; De Cuyper et al., 1983; but see Kremer et al. (1977)], cholesterol exchange between cells in culture and acceptors in the medium (Phillips et al., 1980; Rothblat & Phillips, 1982), and cholesterol exchange between lipoproteins (Lund-Katz et al. 1982) and between red blood cells and lipoproteins (Bojeson, 1982; Lange et al., 1983) proceed through the aqueous phase. Other reports have demonstrated an aqueous diffusion mechanism for exchange of fluorescently

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labeled phospholipids between vesicles (Doody et al., 1980; Roseman & Thompson, 1980; Nichols & Pagano, 1981; Frank et al., 1983). The overall mechanism of exchange involves the desorption of lipid molecules from the donor bilayer into the aqueous phase where the lipid molecules are taken into the acceptor vesicles by collisions between the lipid molecules in the aqueous phase and the acceptor vesicles surface. In a well-defined experimental system consisting of unilamellar lipid vesicles, in the absence of protein, the rate-limiting step for the overall exchange process is desorption (McLean & Phillips, 1981).

The relative rates of exchange of different lipids have been the subject of active investigation. A relatively slow rate of phosphatidylcholine exchange between red blood cells and plasma or lipid vesicles has been observed ($t_{1/2} \sim 60$ h; Hahn & Hevesy, 1939; Sakagami et al., 1963; Soula et al., 1967; Reed et al., 1968; Bloj & Zilversmit, 1977 while the rate of exchange of lysophosphatidylcholine is far greater (Sakagami et al., 1963; Minori & Zilversmit, 1963; Soula et al., 1967). Recent experimental evidence indicates that the rate of exchange of phospholipids is also sensitive to the length of the acyl chain: decreasing the length of the acyl chain increases the rate of exchange markedly [Martin & MacDonald, 1976; Duckwitz-Peterlein et al., 1977; Thilo, 1977; Massey et al., 1982; but see Kremer et al. (1977)]. Thilo (1977) has proposed, using a kinetic model, that the rate of lipid exchange may be predicted on the basis of the aqueous-phase solubility of the lipid. Additional factors such as the composition (McLean & Phillips, 1982) and the physical state (Doody et al., 1980; Frank et al., 1983) of the donor bilayer also influence the rate of lipid exchange.

Here we apply the model for kinetics of micellization developed by Aniansson et al. (1976) to the rate of lipid exchange between unilamellar vesicles. The free energy of activation is found to exceed the free energy of lipid transfer by an amount that can, in part, be attributed to restriction of the lipid molecule to the surface of the donor vesicle in the transition-state complex. The dependence of DMPC exchange rates on the physical state of the donor bilayer is also discussed.

Experimental Procedures

Materials. Dimyristoylphosphatidylcholine (DMPC)[1] and dipalmitoylphosphatidylcholine (DPPC) were purchased from Sigma (St. Louis, MO); egg yolk lysophosphatidylcholine was from Calbiochem-Behring Corp. (LaJolla, CA); dimyristoyl[1-^{14}C]phosphatidylcholine, dipalmitoyl[1-^{14}C]phosphatidylcholine, and lysopalmitoyl[1-^{14}C]phosphatidylcholine were from Amersham (Arlington Heights, IL); dimyristoyl[9,10-^{3}H]phosphatidylcholine, cholesteryl[1-^{14}C]oleate, dipalmitoyl[9,10-^{3}H]phosphatidylcholine, and [1,2,6,7-^{3}H]cholesteryl oleate were from New England Nuclear (Boston, MA). In some experiments, [7-^{3}H]cholesteryl oleate synthesized as described previously (Lentz et al., 1975; McLean & Phillips, 1981) was used. Before incubation, all of the radiolabeled lipids were >98% pure as judged by thin-layer chromatography. In some cases, radiolabeled lipids were purified by TLC before use. After incubation of radiolabeled lipid-containing unilamellar vesicles for 2 h at 37 °C, >97% of the labeled lipids cochromatographed with unlabeled standards on silica gel G thin-layer plates developed in chloroform–methanol–water (95:35:4 v/v). All of the unlabeled lipids were >98% pure after incubation and analysis by TLC. The sources and purities of other materials used are described elsewhere (McLean & Phillips, 1981, 1982).

Preparation and Separation of Unilamellar Vesicles. Small unilamellar vesicles were prepared in 20 mM sodium phosphate, pH 6.0, by the procedure of Barenholz et al. (1977). The size distribution of these vesicles has been described in detail elsewhere (McLean & Phillips, 1981, 1982). Neutral vesicles were separated from negatively charged vesicles containing 15 mol % dicetyl phosphate on short columns of DEAE-Sepharose (Pharmacia) by a modification of the procedure of Hellings et al. (1974) and van den Besselaar et al. (1975) as described previously (McLean & Phillips, 1981). The donor vesicles were prepared with a trace of radiolabeled exchangeable lipid; the neutral vesicles contained [7-^{3}H]cholesteryl oleate as a nonexchangeable marker. Typically, <1% of the negatively charged and 80–90% of the neutral vesicles were recovered in the column eluate.

Kinetic Analysis. For the kinetics of PC exchange, two limiting cases were considered: (1) exchange from both inner and outer monolayers and (2) exchange solely from the outer monolayer as a single pool of radiolabeled PC molecules. In both cases, the kinetic scheme is that of an isotope exchange reaction (McKay, 1938; McLean & Phillips, 1981):

$$kt = -X_{\infty} \ln (1 - X/X_{\infty}) \qquad (1)$$

where k is the frequency of transfer of a lipid molecule from the donor bilayer to the acceptor vesicles, X is the fraction of label transferred to the acceptor vesicle population at time t, and X_{∞} is the fraction of labeled molecules in the acceptor vesicle compartment at $t = \infty$. When all of the labeled molecules exchange rapidly, $X_{\infty} = a/(a + b)$, where a is the concentration of PC in the acceptor vesicle compartment and b is the concentration of PC in the donor vesicle compartment.

The thermodynamic functions associated with formation of the transition-state complex were calculated by using the model of Aniansson et al. (1976) for the kinetics of micellization. Briefly, this model applied to lipid exchange considers the desorbing lipid molecule as a rigid rod of length l moving along its long axis at right angles to the surface of the lipid vesicle surface. The motion is diffusional and is associated with a potential energy $V(X)$ that varies with X, the length of the molecule that resides outside the vesicle. When $X = l$, $V(X)$ reaches a maximal value, here identified with the activation free energy $\Delta G^{\ddagger}$. From eq 48 and 50 of Aniansson et al. (1976) (where $k = k_{s^-}/s$)

$$k = D_{\mathrm{m}}[\Delta G^{\ddagger}/(RTl)]^2 \exp[-\Delta G^{\ddagger}/(RT)] \qquad (2)$$

where D_{m} is the diffusion coefficient for the desorbing lipid molecule. D_{m} is close to the free diffusion coefficient and is calculated from the Stokes–Einstein equation $D = kT/(6\pi\eta r)$, where η is the viscosity of the liquid medium and $2r$ is the long axis length of the diffusing species. An expression for $\Delta H^{\ddagger}$ (activation free enthalpy) is obtained in terms of $\Delta G^{\ddagger}$ and the Arrhenius activation energy (E_{a}) by differentiation of the logarithm of eq 1 with respect to temperature, with the substitutions $\Delta G^{\ddagger} = \Delta H^{\ddagger} - T\Delta S^{\ddagger}$ and $E_{\mathrm{a}} = (d \ln k/\mathrm{d}T)/RT^2$ (Glasstone et al., 1941):

$$\Delta H^{\ddagger} = E_{\mathrm{a}}\Delta G^{\ddagger}/(\Delta G^{\ddagger} - 2RT) \qquad (3)$$

Equation 3 was used to calculate $\Delta H^{\ddagger}$ from values of $\Delta G^{\ddagger}$ obtained from eq 2 and from E_{a} obtained from Arrhenius plots of kinetic data over a range of temperatures. For the activation entropy, $\Delta S^{\ddagger} = (\Delta H^{\ddagger} - \Delta G^{\ddagger})/T$.

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[1] Abbreviations: DMPC, dimyristoylphosphatidylcholine; DPPC, dipalmitoylphosphatidylcholine; PC, phosphatidylcholine; DEAE, diethylaminoethyl; POPC, 1-palmitoyl-2-oleoylphosphatidylcholine; LPPC, lysopalmitoylphosphatidylcholine; TLC, thin-layer chromatography; SEM, standard error of the mean; T_{c}, gel–liquid crystal transition temperature of DMPC.

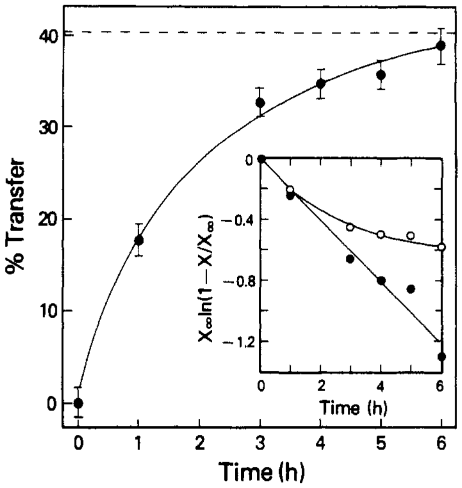

FIGURE 1: DMPC exchange between unilamellar vesicles at 32 °C. Transfer of dimyristoyl-[9,10-^{3}H]PC from unilamellar DMPC vesicles (0.05 mg/mL) containing cholesteryl[1-^{14}C]oleate to unilamellar DMPC vesicles (0.1 mg/mL) containing 15 mol % dicetyl phosphate was followed as described under Experimental Procedures. Each point is the mean ± SEM of six experiments. The dashed line is the maximum percent transfer expected on the basis of exchange from only the outer monolayer of the bilayer (40.4%). The inset is an isotope exchange plot of the average values, assuming a single pool of DMPC [X_∞ = 0.667 (○)] or exchange only from the outer monolayer [X_∞ = 0.404 (●)].

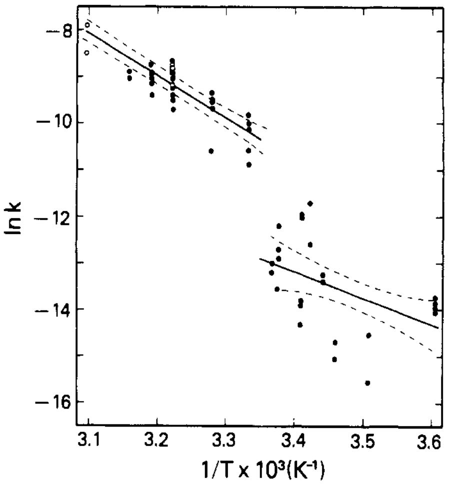

FIGURE 2: Temperature dependence of DMPC exchange. Donor vesicles were composed of DMPC-dimyristoyl-[9,10-^{3}H]PC-cholesteryl[1-^{14}C]oleate (●), 10 mol % cholesterol–90 mol % DMPC–dimyristoyl-[1-^{14}C]PC–[7-^{3}H]cholesteryl oleate (○), 20 mol % cholesterol–65 mol % DMPC–15 mol % dicetyl phosphate–dimyristoyl-[1-^{14}C]PC (■), or 20 mol % cholesterol–65 mol % egg yolk PC–15 mol % dicetyl phosphate–trace amounts of dimyristoyl-[1-^{14}C]PC (□). Acceptor vesicles were composed of 85 mol % DMPC–15 mol % dicetyl phosphate (●), 10 mol % cholesterol–65 mol % DMPC–15 mol % dicetyl phosphate (○), or egg yolk PC–[7-^{3}H]-cholesteryl oleate (■, □). Vesicles were incubated at the indicated temperatures and separated on ion-exchange columns at intervals, and the rate constants were calculated as described under Experimental Procedures. The regression lines are based on dimyristoyl-[9,10-^{3}H]PC exchange between pure DMPC vesicles in the temperature intervals 27–45 °C and 4–24 °C. Each point represents a single experiment. The dashed lines represent the 95% confidence interval of the linear regression line.

Analytical Procedures. Phospholipid concentrations were estimated by analysis for phosphorus (Sokoloff & Rothblat, 1974). Cholesterol was analyzed with the Liebermann and Burchard reagent (Billimoria & James, 1960).

Results

Phosphatidylcholine Exchange. The percentage transfer of radiolabeled DMPC from neutral DMPC vesicles to negatively charged DMPC vesicles as a function of time at 32 °C is given in Figure 1. In each of the experiments summarized in the figure, the ratio of weights of acceptor to donor vesicles was 2:1. If all of the labeled DMPC molecules exchange as a single kinetic pool (case 1), X_∞ = 0.667 in eq 1. Alternatively, when exchange between the outer monolayers of the donor and acceptor vesicles is much more rapid than exchange of molecules between the inner and outer monolayers of a bilayer (case 2), only a fraction (F) of the labeled molecules is available for rapid exchange. Therefore, $X_\infty = F \times 0.667$ in eq 1. An estimate of the fraction of lipid in the outer monolayer was obtained by assuming that the molecular volumes of the average lipids in the inner and outer monolayers of the vesicle bilayer are identical (Huang & Mason, 1978). If the data for DMPC vesicles at 30 °C from Watts et al. (1978) are used, the ratio of outer to inner monolayer volume is 1.54 [cf. de Kruijff & van Zoelen (1978)]. Therefore, the fraction (F) of lipid in the outer monolayers is 0.606, and exchange between only the outer monolayers gives X_∞ = 0.404. The DMPC exchange data are plotted in the inset to Figure 1 with eq 1 and X_∞ = 0.667 and 0.404. The best linear fit to the data is obtained for X_∞ = 0.404, giving a single rate constant, $k = (7.2 \pm 1.6) \times 10^{-5}$ s^{-1}, corresponding to a half-time of 4.0 ± 1.2 h (n = 6) at 32 °C. Exchange solely from the outer monolayer of the vesicle was assumed for all subsequent calculations of PC exchange rates [cf. de Kruijff & van Zoelen (1978)]. At temperatures above 32 °C, over the course of a 6-h incubation, a rapid release of ~40% of the lipid is observed followed by a slow release of additional lipid. The initial, rapid release is identified with the outer monolayer pool of lipid and was used to calculate rate constants. The half-time for DMPC exchange at 37 °C is 2.0 h [cf. de Kruijff & van Zoelen (1978) and De Cuyper et al. (1983)].

The temperature dependence of DMPC exchange from neutral DMPC vesicles is shown in Figure 2 as an Arrhenius plot. Included in this figure are data for DMPC exchange from donors composed of (1) 10 mol % cholesterol–90 mol % DMPC, (2) 20 mol % cholesterol–65 mol % DMPC–15 mol % dicetyl phosphate, and (3) 20 mol % cholesterol–65 mol % egg yolk PC–15 mol % dicetyl phosphate–trace amounts of dimyristoyl[1-^{14}C]PC. Inclusion of cholesterol or dicetyl phosphate (conferring a negative charge to the donor vesicles) or replacement of DMPC by an unsaturated PC mixture (egg yolk PC) does not significantly affect the DMPC exchange rates at 37 °C.

A discontinuity in the Arrhenius plot bewteen 24 and 27 °C is evident in Figure 2 [cf. Doody et al. (1980) for 9-(3-pyrenyl)nonanoic acid transfer between DMPC vesicles]. The temperature range of the discontinuity corresponds to the gel to liquid-crystal phase transition temperature of DMPC multilamellar vesicles [24.5 °C, Phillips (1972)]. We find that bilayers in multilamellar vesicles and small unilamellar vesicles of DMPC are only completely liquid crystalline at 24 °C; the transition in multilamellar vesicles is isothermal whereas the transition is broadened and occurs several degrees lower when small unilamellar vesicles are heated [cf. Suurkuusk et al. (1976) and Gaber & Sheridan (1982)]. The data in Figure 2 show that from 27 to 45 °C the activation energy for DMPC exchange is 70 ± 11 kJ/mol (n = 29); from 4 to 24 °C, the activation energy is 47 ± 19 kJ/mol (n = 24).

In order to establish the relative rates of exchange of DMPC and DPPC from a bilayer of a similar structure, the rate of transfer of a trace amount of radiolabeled DPPC from DMPC donors was followed at 37 °C. DMPC and DPPC mix ideally

Table I: Lysopalmitoylphosphatidylcholine Exchange between Unilamellar Vesicles[a]

donor vesicles	incubation time (min)	% transfer	% recovery of acceptor vesicles
<0.1 mol % lyso-PC	0	31.0 ± 0.6 (8)	92.8 ± 1.5
	10	32.2 ± 0.7 (8)	94.3 ± 0.7
	60	32.4 ± 1.1 (8)	94.9 ± 1.0
	1440	39.4 ± 0.6 (4)	86.3 ± 0.2
5 mol % lyso-PC	0	34.5 ± 0.5 (6)	102.0 ± 1.4
	10	33.7 ± 0.8 (6)	100.1 ± 0.8

[a] Donor vesicles (0.14 mg/mL) contained the indicated mol percent of lyso-PC, 15 mol % dicetyl phosphate and egg PC, and 0.3 μCi of lysopalmitoyl[1-^{14}C]phosphatidylcholine/mg of PC. Acceptor vesicles (1.27 mg/mL) contained egg PC and 0.1 μCi of [7-^{3}H]cholesteryl oleate/mg of PC. Vesicles were incubated at room temperature and separated as described under Experimental Procedures. The percent transfer of lyso-PC is the percent of initial cpm eluted with acceptors from the column; percent recovery is based on recovery of ^{3}H in the eluate. The numbers are ± SEM with the number of experimental points in parentheses.

Table II: Thermodynamic Parameters of DMPC and Cholesterol Exchange[a]

parameter	DMPC $T > T_c$[b]	DMPC $T < T_c$[c]	DMPC Δ[d]	cholesterol[e] at 37 °C
$k \times 10^6$ (s^{-1})	29.6	2.3	12.7	89.4
$\Delta G^\ddagger$ (kJ mol^{-1})	87	94	−7	90
$\Delta H^\ddagger$ (kJ mol^{-1})	75	49	26	77
$\Delta S^\ddagger$ (J mol^{-1} K^{-1})	−42	−149	−107	−41

[a] Parameters were calculated from k values extrapolated to 24.5 °C by the Arrhenius equation with the theory of Aniansson et al. (1976) as described under Experimental Procedures. [b] Data extrapolated to 24.5 °C from data obtained at 27–45 °C. [c] Data extrapolated to 24.5 °C from data obtained at 4–24 °C. [d] For k, the quotient (column 1/column 2); for the remainder, the difference (column 1 − column 2). [e] Cholesterol values are at 37 °C and are calculated from data in McLean & Phillips (1981).

in lipid bilayers, and the concentration level of DPPC (<0.1 mol %) used in these experiments was too low to alter the phase-transition temperature of the vesicles (Phillips et al., 1970); at 37 °C, the vesicles are in the liquid-crystalline phase. In 22 experiments, the average $t_{1/2} = 83 + 12$ h, some 40 times slower than DMPC exchange from similar donors [cf. Martin & MacDonald (1976)]. Analogous decreases in desorption rate with addition of methylene groups have also been observed in other homologous series (Duckwitz-Peterlein et al., 1977; Thilo, 1977; Massey et al., 1982; Pownall et al., 1983).

Lysophosphatidylcholine Exchange. Data for the percentage transfer of lysopalmitoyl[1-^{14}C]phosphatidylcholine from negatively charged egg PC donor vesicles, with or without added lyso-PC, to neutral egg PC acceptor vesicles are given in Table I. In these experiments, the acceptor vesicles were in 10-fold excess so that 90.9% of the label is expected to transfer at infinite time. The data show a rapid transfer of 30–40% of the radiolabeled lyso-PC and no substantial increase in transfer with time over 24 h. The zero time corresponds to immediate passage of the vesicle mixture over the column after a 30-s period of vortexing. Complete separation of donor and acceptor vesicles is achieved in <2 min.

When the donor vesicles contain 5 mol % lyso-PC, 1.0 μg of lyso-PC is applied to the columns used for separation of charged and neutral vesicles. At equilibrium, 0.09 μg is expected to associate with the donor (charged) vesicles bound to the column. However, 66% (0.66 μg) remains with the column; this amount does not change with time (Table I). If 0.66 μg of lyso-PC dissolved in buffer is applied to the column and washed off with 1 mL of buffer, >90% of the lyso-PC adsorbs to the column. Thus, part of the lyso-PC that remains with the column has adsorbed to it. Low concentrations of cholesterol and egg yolk PC also adsorb to the column, but the rate of transfer of these molecules over the 2-min separation period is too slow to influence the kinetic results. From these data, an upper limit of 2 min may be placed on the half-time of lyso-PC transfer to the column and acceptor vesicles [cf. $t_{1/2}$ = 50–500 ms for lyso-PC incorporation from solution into DMPC and DPPC vesicles; Elamrani & Blume (1982)].

Cholesterol Exchange from Lyso-PC-Containing Vesicles. In order to test the effect of lyso-PC incorporated in a PC bilayer on the rate of lipid exchange, [4-^{14}C]cholesterol transfer from lyso-PC-containing vesicles was followed. Cholesterol exchange at 37 °C between 10 mol % cholesterol–egg PC vesicles without lyso-PC proceeds with $k = (1.63 \pm 0.13) \times 10^{-4}$ s^{-1} ($n = 10$) while for similar vesicles that contain 5 mol % lyso-PC, $k = (1.55 \pm 0.17) \times 10^{-4}$ s^{-1}. There is no significant difference in the two rate constants. Consistent with these data, lyso-PC has been shown to have negligible effects on bilayer structure at low concentrations in PC vesicles (Klopfenstein et al., 1974; Van Echteld et al., 1981).

Thermodynamic Parameters of Lipid Exchange. (*a*) *Effect of Lipid Phase Transition.* The thermodynamic parameters of the activated state for DMPC exchange calculated with eq 2 and 3 are shown in Table II. All values are extrapolated from the Arrhenius equation to 24.5 °C, the phase-transition temperature of the unilamellar DMPC vesicle donors. The difference in the thermodynamic parameters above and below the lipid phase transition is also given [cf. Doody et al. (1980) for 9-(3-pyrenyl)nonanoic acid transfer]. None of the thermodynamic parameters calculated from $T > T_c$ data extrapolated to 24.5 °C differ from the value calculated at 37 °C for DMPC exchange. Therefore, the DMPC exchange data for $T > T_c$ may be directly compared with the thermodynamic parameters for cholesterol exchange from liquid-crystalline bilayer vesicles at 37 °C in Table II.

Table III: Rates of Lipid Exchange between Small Unilamellar Vesicles at 37 °C[a]

donor vesicles	$k \times 10^6$ (s^{-1})	$t_{1/2}$ (h)	ΔG_t (kJ mol^{-1})	$\Delta G^\ddagger$ (kJ mol^{-1})[g]	$\Delta G^\ddagger - \Delta G_t$
LPPC–egg PC[b]	>5000 (8)	<0.05	41	<76	<36
DMPC–DMPC[c]	107 ± 12 (9)	2.0	58	87	30
cholesterol–egg PC[d]	89 ± 11 (5)	2.3	56	90	34
POPC–egg PC[e]	3.7 ± 1.2 (8)	63	68	96	27
DPPC–DMPC[f]	3.3 ± 0.4 (22)	83	66	96	30

[a] Values are averages ± SEM for the number of experiments shown in parentheses. The free energies of transfer, ΔG_t, are calculated as described under Results. [b] Palmitoyl-[1-^{14}C]lyso-PC transfer from 15 mol % dicetyl phosphate–85 mol % egg yolk PC donor vesicles. [c] Dimyristoyl-[9,10-^{3}H]PC transfer from DMPC donor vesicles. [d] [4-^{14}C]Cholesterol transfer from 20 mol % cholesterol–15 mol % dicetyl phosphate–65 mol % egg yolk PC donor vesicles (McLean & Phillips, 1981). [e] 1-Palmitoyl-2-oleoyl[1-^{14}C]PC transfer from 20 mol % cholesterol–15 mol % dicetyl phosphate–65 mol % egg yolk PC donor vesicles. [Recalculated from McLean & Phillips (1981) as described under Results.] [f] Dipalmitoyl-[1-^{14}C]PC transfer from DMPC donor vesicles. [g] Free energies of activation calculated as described under Experimental Procedures with the following values (in nm): LPPC and DPPC, $l = 2.0$, $r = 1.5$; DMPC, $l = 1.8$, $r = 1.4$; POPC, $l = 2.1$, $r = 1.5$ (Hauser et al., 1981); cholesterol, $l = 1.6$, $r = 0.8$ (Shieh et al., 1977).

(*b*) *Relationship to Free Energies of Transfer.* The half-times and rate constants for exchange of five different lipids from unilamellar PC vesicles are given in Table III. Transbilayer movement of cholesterol ("flip-flop") is faster than exchange (McLean & Phillips, 1981) so that the rates are based on a single exchangeable pool of cholesterol, while for PC the rates are calculated for exchange from only the outer half of the bilayer vesicle (see Figure 1). The data for POPC exchange (McLean & Phillips, 1981) were recalculated by using a ratio of outer to inner monolayer volume of 1.54. There is no statistically significant difference in the rates of exchange of DMPC and cholesterol, or of POPC and DPPC.

Table III also gives values of ΔG_t, the free energy of transfer of the lipid from its own micelle to water, calculated from the critical micellar concentration (cmc) by $\Delta G_t = -RT \ln(\text{cmc}/55.5)$, which applies for molecules with low aqueous-phase solubilities (Tanford, 1980). Although it is difficult to measure cmc values for the sparingly soluble lipids used in this study, estimates for lysopalmitoylphosphatidylcholine (7×10^{-6} M; Haberland & Reynolds, 1975), cholesterol (12.1×10^{-8} M, extrapolated to infinite dilution; Haberland & Reynolds, 1973), and dipalmitoylphosphatidylcholine (4.6×10^{-10} M; Smith & Tanford, 1972) are available. A value of 1.1×10^{-8} M for DMPC was estimated from the linear relationship between ln cmc and the number of carbons in the PC acyl chain by using data for $n = 7, 8, 10$, and 16 [summarized in Tanford (1980)]. The ΔG_t for POPC was obtained by incrementing the ΔG_t of DPPC by an amount that is identical with the increase in ΔG_t from potassium palmitate to potassium oleate [cmc values of 2.2×10^{-3} M and 4.5×10^{-4} M, respectively (Klevens, 1953)].

Free energies of activation calculated according to eq 2 for each of the lipids are presented in Table III. These free energies of activation are somewhat less than those obtained from the Eyring equation and correspond to transmission coefficients of ~0.01 in the Eyring equation for reactions in solution (Glasstone et al., 1941). In all cases, the activation free energy exceeds the free energy of transfer; the average excess free energy is 30 ± 1 kJ mol^{-1} when the estimated value for the rate of LPPC exchange is excluded.

Discussion

In this paper, the rate of exchange of three classes of biological lipids (PC, lyso-PC, and cholesterol) between unilamellar vesicles was measured in the absence of fusion and protein. In this simple model system, the rate-limiting step for lipid exchange or transfer is desorption of the lipid molecule from the donor bilayer into the aqueous phase (McLean & Phillips, 1981). Two features of this exchange process have been examined in some detail: (1) the effect of the lipid phase transition on the thermodynamic parameters of DMPC exchange and (2) the relationship between the aqueous-phase solubility of the diffusing lipid molecule and the free energy of activation for exchange.

Effect of Lipid Phase Transition on Rates of Exchange. The data of Figure 2 clearly demonstrate that the rate of desorption of DMPC from DMPC bilayers depends on the physical state of the donor lipid bilayer. At temperatures above the phase-transition temperature (T_c) of the unilamellar vesicles, where the DMPC is in a liquid-crystalline phase, the rapid rate and high activation energy for exchange are similar to those for cholesterol transfer out of an egg PC bilayer. At temperatures below T_c, the lipid is in the gel phase, and DMPC exchange proceeds relatively slowly and with a lower activation energy [cf. Doody et al. (1980) and Massey et al. (1982)]. When the data for DMPC exchange above and below T_c are extrapolated to T_c, the rate of exchange from the liquid-crystalline phase is approximately 1 order of magnitude greater than that from the gel phase. In contrast, the rate of cholesterol exchange from DMPC vesicles is not influenced significantly by the physical state of the donor vesicle lipid (McLean & Phillips, 1982). This difference in behavior arises because cholesterol exchange proceeds from a cluster of cholesterol and PC molecules that are laterally phase separated from free-melting PC; this cholesterol-rich region of the bilayer does not sense the phase transition of the remaining DMPC molecules. On the other hand, radiolabeled DMPC is randomly distributed within the DMPC bilayer, and its rate of exchange is sensitive to the surrounding PC, which undergoes a phase change at T_c.

The values for the activation entropy and enthalpy calculated from the DMPC exchange data with the theory of Aniansson and co-workers (1976) are compared above and below T_c in Table II. The differences between ΔH^* and ΔS^* above and below T_c compare favorably with the ΔH and ΔS values associated with the phase transition of fully hydrated DMPC measured by differential scanning calorimetry [$\Delta H = 27.9$ kJ mol^{-1}, $\Delta S = 94.1$ J mol^{-1} K^{-1}; Phillips (1972)]. It follows that the same ΔH and ΔS values are involved in moving from the transition state below T_c to the transition state above T_c as are associated with moving from the donor bilayer below T_c to the bilayer above T_c. This suggests that the conformation of the hydrocarbon chains of the desorbing molecule when in the donor bilayer is maintained as the molecule moves to the transition state (i.e., the transition state below T_c involves the all-trans conformation whereas gauche conformers are involved above T_c).

Interpretation of the magnitudes of ΔH^* and ΔS^* necessitates a better understanding of the interactions of desorbing lipid molecules with water molecules in the interfacial water region [cf. Massey et al. (1982)]. However, both above and below T_c desorption is associated with an increase in enthalpy and a decrease in entropy of formation of the transition-state complex (Table II). Similarly, for cholesterol exchange, the enthalpy of activation is positive and the entropy of activation is negative. The close correspondence between the thermodynamic parameters of activation for DMPC exchange and cholesterol exchange from liquid-crystalline bilayers suggests that similar physical events lead to formation of the transition-state complex and that the mechanism of exchange of DMPC and cholesterol is identical.

Effect of Aqueous-Phase Solubility on Rates of Exchange. A close correspondence between the aqueous-phase solubility of a lipid molecule and its exchange rate is evident for exchange of various lipids between unilamellar vesicles (Table III). Both the free energy of transfer and the activation free energy for DMPC and DPPC are greater for the higher homologue. The increases per methylene group are 2.1 and 2.2 kJ mol^{-1} for ΔG_t and ΔG^*, respectively. These values are consistent with the expected change in hydrophobic free energy as methylene groups are added to an acyl chain [cf. Tanford (1980)], indicating that both ΔG_t and ΔG^* are determined by the hydrophobic effect. For all the systems described in Table III, the difference $\Delta G^* - \Delta G_t \sim 30$ kJ mol^{-1} [cf. Israelachvilli et al. (1980)]. This difference may be used to estimate a value of ΔG^* from the cmc of the lipid. Substitution of this estimated ΔG^* into eq 1 gives a predicted value of $t_{1/2} = 21$ s for LPPC exchange in agreement with the experimental value of $t_{1/2} < 2$ min.

The additional free energy, in excess of the free energy of transfer, required to form the transition-state complex may

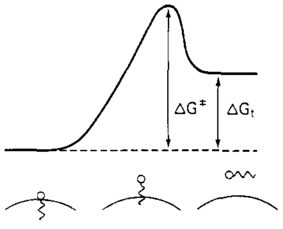

FIGURE 3: Free-energy diagram of lipid exchange from bilayers. Transfer of lipid molecules from the bilayer to the aqueous phase proceeds through a transition-state complex where the lipid molecule is attached to the vesicle by the tip of its hydrophobic tail. Formation of this activated-state complex is associated with a high energy of activation, which exceeds the free energy of transfer by an amount that is determined, in part, by restriction of the lipid molecule to the surface of the bilayer vesicle.

be understood by reference to Figure 3 [cf. Aniansson et al. (1976)]. The highest free-energy state along the reaction coordinate is a lipid molecule almost completely transferred to the aqueous phase but still associated with the vesicle surface by the tip of its hydrophobic tail. Such a structure would have a lower entropy than the lipid within the bilayer as a result of the hydrophobic effect. A decrease in entropy associated with formation of the transition-state complex is consistent with the data in Table II. When the tip of the hydrophobic tail leaves the vesicle surface, the free energy of the system decreases due to increased freedom of the monomer to tumble and diffuse in three dimensions. For the systems examined here, this decrease in free energy amounts to ~30 kJ mol^{-1}.

Transfer of the lipid molecule from the vesicle interface (transition-state complex) to the aqueous phase results in an increase in one translational and two rotational (long axis) degrees of freedom in the lipid molecule. An estimate of the free energy required to restrict the lipid molecule to the vesicle surface may be obtained by using the methods of statistical thermodynamics (Hill, 1960). By use of a model developed by Janin (1978) the total free-energy increase associated with immobilization of a molecule the size and density of cholesterol at 20 °C is 42 kJ mol^{-1} for three translational degrees of freedom and 41 kJ mol^{-1} for three rotational degrees of freedom. Assuming equipartition of the free-energy changes among the degrees of freedom, the loss of two rotational and one translational degrees of freedom is 41 kJ mol^{-1}. It is tempting to identify this 41 kJ mol^{-1} increase in free energy due to a configurational restriction of the free lipid molecule with the ~30 kJ mol^{-1} difference between the free energy of activation and the free energy of transfer. At least three sources of the discrepancy between the two values may be identified: (1) the ideal gas assumption used in deriving the equations, (2) the possible contributions of altered C–C rotational mobility, and (3) inadequate estimates of the free energy of transfer of lipid molecules between bilayers and water. Point 3 may be particularly important for DPPC in DMPC bilayers and cholesterol in egg PC bilayers. The correspondence must remain semiquantitative until a more complete thermodynamic analysis of the transition-state complex is elicited.

Physiological Significance. The relationship between ΔG_t and $\Delta G^{\ddagger}$ in Table III provides a basis for calculation of estimates (from eq 1) of the expected $t_{1/2}$ value for transfer of lipids where the aqueous-phase solubility (i.e., a value for ΔG_t) of the lipid is known. For molecules such as cholesteryl esters and triglycerides the $t_{1/2}$ for exchange would be expected to be >100 h at 37 °C. Such low rates of spontaneous lipid exchange are apparently inadequate in vivo, and lipid exchange proteins that promote the rate of exchange of sparingly soluble lipids are commonly found for PC, cholesteryl esters, and triglycerides [see, e.g., Wirtz (1974) and Barter & Jones (1980)]. For 25-hydroxycholesterol [cmc ~ 2×10^{-6} M; Sinensky (1981)], the predicted $t_{1/2}$ = 0.6 s. Such a rapid exchange may be important in the metabolic role of certain oxidized sterols in regulation of cellular cholesterol metabolism.

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Registry No. DMPC, 13699-48-4; POPC, 6753-55-5; DPPC, 2644-64-6; cholesterol, 57-88-5; diacetyl phosphate, 3614-36-6; cholesteryl oleate, 303-43-5.

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Effects of Heating on the Ion-Gating Function and Structural Domains of the Acetylcholine Receptor[†]

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ABSTRACT: The ion-gating ability and the protein electrophoretic band patterns of the acetylcholine receptor from *Torpedo californica* electroplax were examined after receptor-enriched membrane vesicles were progressively heated. The ion translocation function was lost over a temperature range of 40–55 °C. Previous results have shown that the stoichiometry of α-bungarotoxin binding is not affected by these temperatures, although bound toxin reversibly dissociates within this temperature range, and that toxin binding is irreversibly lost at somewhat higher temperatures [Soler, G., Farach, M. C., Farach, H. A., Jr., Mattingly, J. R., Jr., & Martinez-Carrion, M. (1983) *Arch. Biochem. Biophys. 225*, 872]. Thermal gel analysis [Lysko, K. A., Carlson, R., Taverna, R., Snow, J., & Brandts, J. F. (1981) *Biochemistry 20*, 5570], a sodium dodecyl sulfate–polyacrylamide gel electrophoretic procedure which detects thermally induced aggregation of the components of multimeric systems, was applied to heated acetylcholine receptor enriched membranes. This technique suggests two structural domains susceptible to thermal perturbation within the receptor molecule, one consisting of the M_r 50 000 and the two M_r 40 000 subunits and the other consisting of the M_r 60 000 and 65 000 subunits. Heat disrupts molecular events linking agonist binding with ion-channel opening in the acetylcholine receptor molecule.

The abundance of acetylcholine receptor (AcChR)[1] in the electric tissue of *Torpedo californica* has allowed significant progress in the understanding of the structure of this ion-gating, transmembrane protein. The elementary functional unit of the AcChR is a pentameric protein with an $\alpha_2\beta\gamma\delta$ stoichiometry (Reynolds & Karlin, 1978) with apparent subunit molecular weights of 40 000, 50 000, 60 000, and 65 000 and with an overall molecular weight of 270 000 (Martinez-Carrion et al., 1975). In vivo, these elementary units appear to exist as dimers linked by a disulfide bond between δ subunits

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[1] Abbreviations: AcChR, acetylcholine receptor; DSC, differential scanning calorimetry; α-Bgt, α-bungarotoxin; CARB, carbamylcholine; SDS, sodium dodecyl sulfate; IgG, immunoglobulin G; EDTA, ethylenediaminetetraacetic acid; PMSF, phenylmethanesulfonyl fluoride; HEPES, N-(2-hydroxyethyl)piperazine-N'-2-ethanesulfonic acid; DEAE, diethylaminoethyl; PAGE, polyacrylamide gel electrophoresis; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; NEM, N-ethylmaleimide.

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