Kinetics of Symmetric and Asymmetric Phospholipid Transfer between Small Sonicated Vesicles Studied by High-Sensitivity Differential Scanning Calorimetry, NMR, Electron Microscopy, and Dynamic Light Scattering†

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Received January 7, 1988; Revised Manuscript Received April 4, 1988

ABSTRACT: The transfer of L_α phosphatidylcholines of different acyl chain length between small unilamellar vesicles via the aqueous phase was studied by using high-sensitivity differential scanning calorimetry (hs-DSC), ^{1}H NMR, dynamic light scattering, and freeze-fracture electron microscopy. Two cases of lipid transfer were studied: (1) the asymmetric lipid transfer between phosphatidylcholine vesicles with different acyl chain lengths of the lipids; (2) the symmetric lipid transfer between phosphatidylcholine vesicles of the same type of lipid by using acyl chain deuteriated and protonated lipid analogues. From the hs-DSC data the off rates of the lipids were calculated for both types of transfer as a function of incubation temperature of the vesicles and the acyl chain lengths of the lipids by using a kinetic model. Significant differences between the two types of lipid transfer were found. In the asymmetric case a net transfer of lipids from the vesicle fraction with the lower lipid chain melting temperature (donor vesicles) to those with the higher one (acceptor vesicles) was observed even while the latter were in the gel state. Additionally, the transfer kinetics was strongly dependent on the proportion between donor and acceptor vesicles. In the symmetric case the lipid transfer between the two vesicle populations was nearly equal (i.e., lipid transfer in a 1:1 ratio between the vesicles) and occurred only if all lipids were in the liquid-crystalline state. A further characteristic of the asymmetric lipid transfer was an acceleration of the flip-flop rate of the acceptor vesicles by at least 1 order of magnitude in the initial stage of transfer. The dependence of the transfer kinetics on the incubation temperature, the acyl chain lengths of the lipids, and the total lipid concentration was found to be similar for both types of lipid transfer, provided that all lipids were in the fluid state. It is further shown that lipid transfer is a characteristic of small sonicated vesicles, whereas larger vesicles prepared by detergent dialysis exchanged lipids mainly by vesicle fusion.

It has been established in recent years that phospholipids can transfer between bilayer vesicles through the bulk solution with a transfer rate depending on the lipid and the temperature. The methods used to study this transfer can be divided into three classes: (1) fluorescence assays using labeled phospholipids (Roseman & Thompson, 1980; Nichols & Pagano, 1981, 1982; Xü et al., 1983; Silvius et al., 1987); (2) measurement of the concentration of isotopically labeled lipids after incubation of the mixture and separation of the acceptor vesicles (McLean & Philips, 1981; Patton et al., 1985); (3) calorimetric and light scattering methods (Papahadjopoulos et al., 1976; Martin & MacDonald, 1976; Duckwitz-Peterlein et al., 1977; Kremer et al., 1977). The transfer rates as determined by these methods differ considerably. This is mainly due to the following shortcomings of these methods. Fluorescence assays can detect the transfer of the labeled lipid only, which is not representative for the bulk lipid. For the analysis of the transfer of isotopically labeled lipids, a separation by chromatography is required, which can affect the results. Classical scanning calorimetry and light scattering, which require no special probes, lack sensitivity; i.e., a large fraction of lipid (>10%) must be transferred in order to detect changes in the heat capacity curves.

New high-sensitivity differential scanning calorimeters, in the following abbreviated hs-DSC,[1] can overcome the latter disadvantages. Changes in the thermotrophic behavior of vesicles upon mixing with other lipids can be detected at mixing ratios well below 5% at large excess of water (lipid concentration below 0.5 mg/mL). This enables the observation of the initial stage of lipid transfer between vesicles and allows one to distinguish between the outer and the inner monolayer of the vesicles. Thereby, conclusions about the effect of lipid transfer on the transversal diffusion of lipids between the two monolayers of the vesicles can be drawn. This is a crucial point in all lipid transfer processes that has not yet been clarified by the methods cited above.

This study presents results of lipid transfer between sonicated unilamellar phosphatidylcholine vesicles with different acyl chain lengths yielded by high-sensitivity DSC, ^{1}H NMR, dynamic light scattering (DLS), and freeze-fracture electron microscopy. The results demonstrate that the flip-flop rate of the acceptor vesicles is increased during the initial stage of transfer and may become the rate-limiting step in the later stage of transfer. Moreover, the use of phospholipids with perdeuteriated chains enabled us to study the exchange of lipids between vesicles of equal composition. This type of exchange was found to be completely symmetric. In contrast, lipid transfer between vesicles of phosphatidylcholines with different chain lengths was highly asymmetric and implied always a net

† Supported by the BMFT, the BMBW, and the Fonds der chemischen Industrie.

[1] Abbreviations: DLPC, dilauroylphosphatidylcholine; DMPC, dimyristoylphosphatidylcholine; DMPC-d_{54}, dimyristoylperdeuteriophosphatidylcholine; DPPC, dipalmitoylphosphatidylcholine; DPPC-d_{62}, dipalmitoylperdeuteriophosphatidylcholine; DSPC, distearoylphosphatidylcholine; DLS, dynamic light scattering; hs-DSC, high-sensitivity differential scanning calorimetry; T_c, gel to liquid-crystalline transition temperature; T_{inc}, incubation temperature; $R_{o/i}$, outside to inside molar lipid ratio of vesicles.

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transfer of lipids. These differences were confirmed by measurements of the monolayer mass ratios of the vesicles during lipid exchange by ^{1}H NMR. DLS and freeze-fracture electron microscopy were used in order to detect changes in size and structure of the vesicles due to lipid exchange.

The off rates of the lipids dissociated from the vesicles were calculated by using a kinetic model developed by Thilo (1977) following a proposal of Nakagawa (1974) for the description of micelle association and dissociation. The data demonstrate that the initial stage of exchange can be described by this simple model.

Materials and Methods

Vesicle Preparation. The L_α phosphatidylcholines DLPC, DMPC, DPPC, and DSPC were purchased from Sigma and all deuteriated phospholipids from Avanti Polar Lipids. The lipids were checked for purity by thin-layer chromatography before use.

The lipids were dispersed in 50 mM Hepes, pH 7.0, and 2 mM EDTA (buffer I) and incubated at 10 °C above its gel to liquid-crystalline phase transition temperature (T_c) for 3 h. Then the lipid dispersions were sonicated at this temperature by using a Branson tip sonifier (15 min at 30 W, pulsed mode with 50% duty cycle). In order to remove lipid aggregates and titanium dust, the vesicles were ultracentrifuged at 150000g for 1 h and finally incubated at 10 °C above their T_c for again 8 h.

Vesicles of equimolar DMPC/DPPC composition were prepared by dissolving the lipid mixture in chloroform. The solvent was evaporated under a stream of nitrogen followed by overnight vacuum desiccation. The lipid film was taken up in buffer I and further processed as described above.

For some experiments vesicles were prepared by sodium deoxycholate dialysis in a 50 mM Tris-HCl buffer, pH 7.6, following the procedure given by Enoch and Strittmatter (1979).

For ^{1}H NMR measurements a 50 mM $Na^2H_2PO_4$/$Na_2{}^2HPO_4$, pD 7.0, buffer was used instead of buffer I.

hs-DSC Measurements. High-sensitivity DSC measurements were performed with an MC-2 (Microcal, Amherst, MA) microcalorimeter interfaced to an IBM AT microcomputer. The data were stored and analyzed by this computer using the DA-2 software provided by Microcal.

The two types of lipid vesicles were mixed under gentle vortexing in the desired lipid molar ratio r (see eq 9 below for definition of r) at the incubation temperature T_{inc}. This stock solution was incubated at T_{inc} by storage in a thermostated water bath. At various incubation time for hs-DSC measurements, samples (1.5 mL each) were taken from this stock solution. They were transferred to a cooling bath to equilibrate at the starting temperature of the hs-DSC scan for 5 min and then filled into the calorimeter. The heating scan was started after 20 min of additional equilibration with a scan rate of 80 °C/h and a 10-s time increment between each data acquisition. Control measurements were also performed at a 40 °C/h scan rate and gave identical results.

In order to avoid effects of phospholipid hydrolytic breakdown products, the maximum incubation time was 48 h. As was recently demonstrated by Lentz et al. (1987) for DMPC and DPPC vesicles, no hydrolysis product could be detected before 12 days of storage.

The phosphorus contents of the samples were determined by the method of Chen et al. (1956). The typical phospholipid concentration was 0.5–0.6 mg/mL.

The enthalpies of the individual transitions at the beginning of incubation and of the mixed transitions were determined after subtraction of the buffer base line. The integration limits were defined by connecting the regions of flat base line with a straight line. This and the integration were done via a subroutine of the DA-2 program.

The transition temperatures (T_c) of the pure vesicles were obtained from the specific heat maximum of the endotherms. For asymmetric or split line shapes of the composite vesicles the transition temperature (T_c) was defined as that temperature at which the area of the endotherm was divided into two equal halves (cf. the arrows in Figure 1).

In a separate experiment it was established that the substitution of water by heavy water, as required for ^{1}H NMR measurements, had no effects on the endotherms.

Analysis of the hs-DSC Data. The molar fraction of phospholipid taken up and mixed into the respective acceptor vesicles was calculated from the shifted T_c values of the transition endotherms. They were successively measured with increasing incubation time after the two vesicle populations were mixed. Consider two vesicle populations I and II and assume that at time zero ($t = 0$) the former contains lipid A only and the latter contains lipid B only. After an incubation time t, the transition temperatures $T^{I}_{(t)}$ and $T^{II}_{(t)}$ of vesicle populations I and II can be expressed as

$$T^{I}_{(t)} = x^{I}(T^{B}_{c} - T^{A}_{c}) + T^{A}_{c} \quad (1)$$

$$T^{II}_{(t)} = x^{II}(T^{B}_{c} - T^{A}_{c}) + T^{A}_{c} \quad (2)$$

where x^{I} and x^{II} are given by

$$x^{I} = [B]_{I}/([A]_{I} + [B]_{I}) \quad (3)$$

$$x^{II} = [B]_{II}/([A]_{II} + [B]_{II}) \quad (4)$$

the molar fractions of lipid B in vesicle populations I and II, respectively. In the above equations lipid B is taken to be the phospholipid species with the higher T_c value as compared to lipid A ($T^{B}_{c} > T^{A}_{c}$).

From the values of $T^{I}_{(t)}$ and $T^{II}_{(t)}$ at the incubation time t, the off rate constants k^{off}_{A} and k^{off}_{B} of lipid A dissociated from vesicle I and lipid B dissociated from vesicle II, respectively, were calculated according to the method of Thilo (1977). This kinetic model is based on the assumption that the rate at which lipid monomers in the bulk solution are captured by the vesicles is proportional to the product of the monomer concentration, [M], and the total bilayer surface area in a unit volume. As the latter is proportional to the concentration of lipids in the vesicle, [V], one can write

$$d[V]/dt = k^{on}[M][V] - k^{off}[V] \quad (5)$$

Equation 5 implies that other processes such as lipid flip-flop are not rate limiting, which certainly is a good approximation for the initial stage of lipid transfer. Under the assumption that the rate constants k^{on} and k^{off} for each lipid species are independent of the lipid composition of the vesicles, the transition temperatures in eq 1 and 2 are related to the off rate constants k^{off}_{A} and k^{off}_{B} according to

$$T^{I}_{(t)} = \left[\frac{1 - \exp\{-yt\}}{1/r - (1 - k^{off}_{A}/k^{off}_{B})\exp\{-yt\}}\right](T^{B}_{c} - T^{A}_{c}) + T^{A}_{c} \quad (6)$$

$$T^{II}_{(t)} = \left[\frac{(1 - r)k^{off}_{B}\exp\{-yt\} + rk^{off}_{A}}{(1 - r)(k^{off}_{B} - k^{off}_{A})\exp\{-yt\} + k^{off}_{A}}\right] \times (T^{B}_{c} - T^{A}_{c}) + T^{A}_{c} \quad (7)$$

where

$$y = rk^{off}_{A} + (1 - r)k^{off}_{B} \quad (8)$$

and

$$r = [B]/([A] + [B]) \quad (9)$$

In the case of symmetric lipid-exchange experiments, the values of $T^{I}_{(t)}$ and $T^{II}_{(t)}$ were obtained pairwise for various incubation temperatures T_{inc}. Insertion of these values and the corresponding incubation times t into eq 6 and 7 yielded two equations from which k^{off}_{A} and k^{off}_{B} were evaluated for each pair of $T^{I}_{(t)}$ and $T^{II}_{(t)}$. The off-rate constants evaluated for the initial stage of exchange [i.e., for all incubation times t at which the amount of exchanged lipid did not exceed 20% (mol)] for each pair of T^{I} and T^{II} were averaged. The standard deviations of the off rates averaged over the incubation times were lower than 4% (n = 5–8) for all rate constants presented in Table I. As the vesicle populations of lipids A and B are essentially the same in the case of symmetric transfer, it follows that $k^{off}_{A} = k^{off}_{B}$ and y reduces to $y = k^{off}_{A}$, independent of r. Therefore, only k^{off}_{A} is given in Table I for symmetric lipid transfer. The off rates from Table I were used to calculate the theoretical curves (drawn lines in Figures 2, 4, and 5).

For the case of asymmetric transfer the off-rate constant of the lipid species with the higher T_c value was assumed to be zero, which reduced the calculation of k^{off}_{A} to one equation (eq 7). This assumption is justified by the results presented below.

The values for $t_{1/2}$ given in Table I were derived from the hs-DSC data as that time after which 50% (mol) lipid was transferred from the donor to the acceptor vesicle.

NMR Measurements. ^{1}H NMR measurements were performed at 400 MHz with a Bruker AM-400 spectrometer. Vesicle solutions were filled into several NMR tubes directly after mixing. The tubes were incubated at the desired T_{inc}. Samples were measured after different incubation periods. In order to obtain sharp signals the measurements were done at temperatures above the T_c of the higher melting lipid species present in the mixture. Thus, DMPC/DPPC vesicle mixtures were measured at 45 °C and those of DMPC/DMPC-d_{54} at 35 °C. The lipid concentration used in these experiments was identical with that used in hs-DSC measurements (0.5–0.6 mg/mL).

Directly before each measurement, a paramagnetic shift reagent ($EuCl_3$) was added to the sample to a final concentration of 2 mM. The resulting splitting of the choline group protons due to a pseudo contact shift interaction of europium ions with the lipid head groups was used to evaluate the outside/inside lipid ratio, $R_{o/i}$. This was achieved by division of the integral areas of the corresponding peaks (cf. Figure 6).

DLS Measurements. The dynamic light scattering apparatus consisted of a Spectra 164 Arion laser, a homemade goniometer, and EMI 9865 photomultiplier tube, and a Brookhaven BI2020 correlator. Vesicle solutions were transferred after ultracentrifugation into the precleaned glass cells (1-mL sample volume) in a dust-free atmosphere. Vesicle concentrations were identical with those used in hs-DSC and NMR experiments (0.5–0.6 mg/mL). The sample cells were put into a temperature-controlled toluene index matching bath, and the homodyne scattered intensity autocorrelation function was measured at 90° scattering angle with 50–150 mW laser power at 514.5 nm. The temperature was kept at 44 (±0.3 °C). Between measurements, the samples were stored in a thermostated water bath at 45 °C.

The multiplexing facility of the BI2020 was used to obtain an autocorrelation function with 256 data points and a sample time of 2 μs. Data were accumulated for typically 200 s in order to get a base line of the order of 10^6. Analysis of the normalized data was performed on a Cyber computer employing both a constrained inverse Laplace-transform program, CONTIN (Provencher, 1982), and a multiexponential fitting program, DISCRETE (Provencher, 1976; Provencher & Vogel, 1980). In our case CONTIN evaluated the data by assuming a smooth distribution of radii, taking into account form factors for hollow spheres (Provencher, 1982) with a wall thickness of 50 Å.

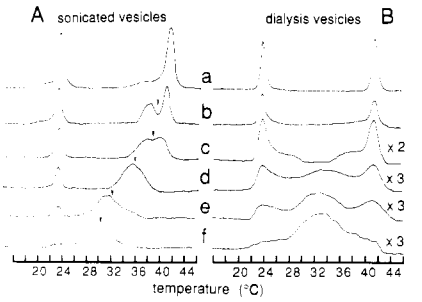

FIGURE 1: hs-DSC endotherms of a 1:1 (mol) mixture of two vesicle populations of DMPC and DPPC prepared by sonication (column A, left, traces a–f) and by detergent dialysis (column B, right, traces a–f) as a function of the incubation time t at 45 °C incubation temperature. Sonicated vesicles: t = 0.1 h (A-a), 0.4 h (A-b), 0.7 h (A-c), 4.5 h (A-d), 18 h (A-e), 28 h (A-d); the arrows indicate the transition temperatures T_c of the composite vesicles obtained from these endotherms according to the convention (cf. Materials and Methods). Dialysis vesicles: t = 0.1 h (B-a), 1.8 h (B-b), 5.5 h (B-c), 16 h (B-d), 28 h (B-e), 47 h (B-f); the traces B-c–f were increased in intensity by the factors given at the right margin.

Electron Microscopy. Freeze-fracture electron micrographs of the samples used for hs-DSC measurements were taken both before the vesicles were mixed and after various incubation times. The samples were frozen by dipping into liquid nitrogen, processed in a Balzers freeze-fracture device, and observed by using a Phillips EM 400T electron microscope.

RESULTS

Asymmetric Lipid Transfer between Vesicles. A good example for a completely asymmetric lipid transfer between vesicles is given by a mixture of two vesicle populations, one of which consists of DMPC and the other of DPPC. The time-dependent changes of the hs-DSC traces of such a mixture, incubated at 45 °C after mixing, is presented in Figure 1A, traces a–f. At the very beginning of the incubation period (t < 0.15 h), the two gel to liquid-crystal transition curves are unaffected by lipid transfer. The peaks show a broad shoulder extending to low temperatures, which is typical for unilamellar vesicles with diameters between 25 and 60 nm, as was recently demonstrated by Lentz et al. (1987). With increasing incubation time, the original transition of the DPPC vesicle population is slightly shifted to lower temperatures, and simultaneously a broad peak appears at lower temperature. This corresponds to a net low temperature shift of T_c (cf. arrows in Figure 1A).

An interesting feature is the initial splitting of the DPPC transition curve into two parts of about equal areas (Figure 1A, trace b). One part remains close to the original position at 41.3 °C, while the other is shifted to a lower temperature. A maximum separation of 2.6 °C was observed 25 min after mixing for the system in Figure 1A. Longer incubation times caused a gradual decrease in intensity and a shift toward lower temperatures of the sharp transition (Figure 1A, trace c) until it coalesced with the broad part of the transition curve. This process was completed 50 min after mixing (Figure 1A, trace d). The coalesced peak was further shifted down to an

Table I: Kinetic Data of Asymmetric and Symmetric Lipid Transfer

vesicle population I (lipid A)	vesicle population II (lipid B)	r = [B]/([A] + [B])	incubation temp (°C)	$t_{1/2}$ (h)	off rate lipid A ($\times 10^5$ s^{-1})
		Asymmetric Lipid Transfer			
DMPC	DPPC	0.5	28	4.8	4.5
DMPC	DPPC	0.5	35	4.5	5.6
DMPC	DPPC	0.25	35	21	2.1
DMPC	DPPC	0.75	35	1.5	14.5
DMPC	DPPC	0.5	45	3.8	9.0
DMPC	DPPC	0.5	55	1.8	22
DMPC	DPPC	0.5	65	0.5	62
DMPC	DPPC	0.5	75	0.2	98
DLPC	DMPC	0.5	35	0.18	120
DPPC	DSPC	0.5	55	41	0.29
		Symmetric Lipid Transfer			
DMPC-d_{54}	DMPC	0.5	35	5.0	4.5
DMPC-d_{54}	DMPC	0.25	35	5.1	4.4
DMPC-d_{54}	DMPC	0.75	35	5.1	4.5
DMPC-d_{54}	DMPC	0.5	55	1.2	17
DPPC-d_{62}	DPPC	0.5	55	46	0.23

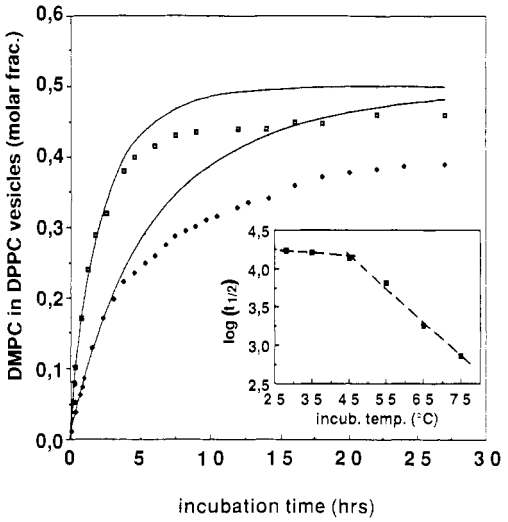

FIGURE 2: Molar fraction of DMPC in DPPC vesicles due to asymmetric DMPC transfer from DMPC to DPPC vesicles versus incubation time at 35 °C (◆) and 55 °C (□) incubation temperature. The drawn lines were calculated according to the kinetic model for the off-rate constants given in Table I. The insert shows a plot of log ($t_{1/2}$) versus the incubation temperature T_{inc}, where $t_{1/2}$ is the half-time for 50% lipid transfer.

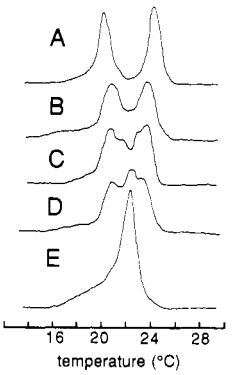

FIGURE 3: hs-DSC endotherms of a 1:1 (mol) mixture of two vesicle populations of DMPC and DMPC-d_{54} for various incubation times at 35 °C incubation temperature: (A) 0.15 h; (B) 3.0 h; (C) 5.0 h; (D) 12.5 h; (E) 25 h.

equilibrium position (Figure 1A, trace f). This corresponds to the endotherm of a homogeneous mixed equimolar DMPC/DPPC vesicle solution. The DMPC peak was neither shifted nor broadened. Its intensity continuously decreased, indicating that no DPPC was mixed into the DMPC vesicles. The small residual bend at the DMPC T_c vanished after some days of incubation.

The enthalpy of the whole thermogram decreased in the initial stage of lipid transfer (0.9 h at 45 °C) from 8.1 to 7.2 kcal/mol. For longer incubations (up to 30 h) the enthalpy was constant at 7.2 ± 0.2 kcal/mol.

From eq 1 the molar fraction of DMPC in DPPC vesicles was calculated. The time course of this ratio is presented in Figure 2. Half-times for 50% lipid transfer and rate constants calculated according to the kinetic model of Thilo (1977) for all measurements studied in this work are given in Table I.

In striking contrast to the behavior of small sonicated unilamellar vesicles was that of a mixture of larger DMPC and DPPC vesicles prepared by dialysis. The diameter of the dialysis vesicles was 200–900 nm as measured by electron microscopy. The endotherms of this mixture, incubated at the same T_{inc} as the sonicated vesicles, are presented in Figure 1B, traces a–f. It can be clearly seen that the original transition temperatures are not shifted. The endotherms broadened considerably with increasing incubation time. A new broad peak grew after about 16 h of incubation time (Figure 1B, trace d), centered between the original peaks and becoming the dominating feature toward the end of the incubation period (Figure 1B, trace f). The integral intensity of the broadened original peaks decreased in a complementary way to the increase of the central peak. The total enthalpy of this thermogram remained constant at 8.4 ± 0.2 kcal/mol over the incubation period.

Symmetric Lipid Transfer between Vesicles. As an example for a symmetric lipid transfer between two vesicle populations, the exchange between DMPC vesicles and DMPC-d_{54} vesicles was studied in detail. The time-dependent changes of its hs-DSC traces after incubation at 35 °C are shown in Figure 3. In the initial stage of exchange both transition curves were equally broadened and shifted toward the center. A salient feature was again a splitting of both transitions at a later stage of exchange (cf. Figure 3C). The two split curves eventually merged into a single but asymmetric peak (Figure 3E). The same asymmetric transition curve was also observed for equimolar homogeneously mixed DMPC/DMPC-d_{54} vesicles with a similar size distribution.

The molar fractions of exchanged lipid due to symmetric exchange versus incubation time t are shown in Figure 4; the kinetic data are presented in Table I. In contrast to the case of asymmetric transfer (Figure 2), the data in Figure 4 can be fitted well by a single exponential. However, as both transition peaks merge in the final stage of symmetric exchange

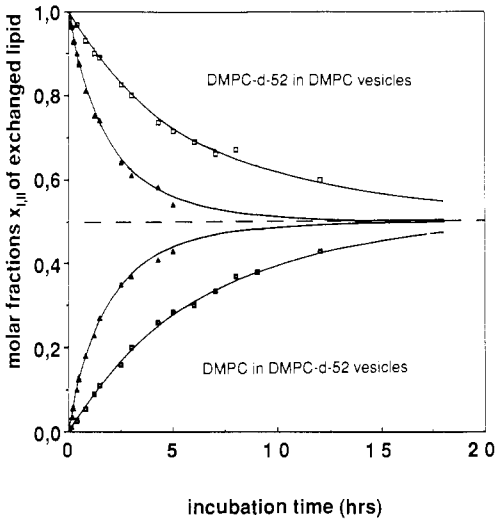

FIGURE 4: Molar fractions x_I and x_{II} of DMPC in DMPC-d_{54} vesicles and DMPC-d_{54} in DMPC vesicles, respectively, during symmetric lipid transfer versus incubation time at 35 °C (□) and 55 °C (△) incubation temperature. The drawn lines were calculated according to the kinetic model by using the corresponding off-rate constants given in Table I.

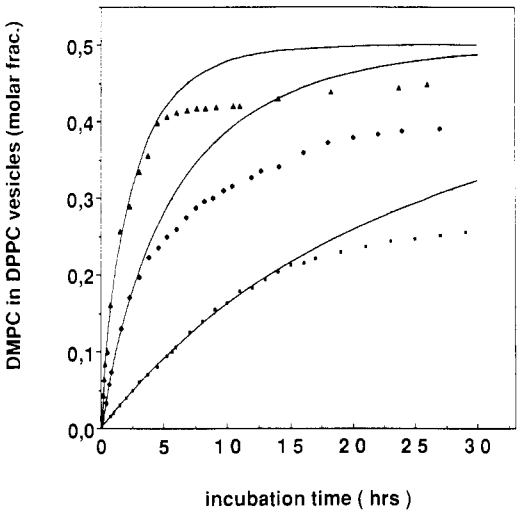

FIGURE 5: Molar fraction of DMPC in DPPC vesicles during asymmetric DMPC transfer from DMPC to DPPC vesicles versus incubation time at various vesicle molar fractions r of DPPC to DMPC vesicles: (■) $r = 0.25$; (◆) $r = 0.5$; (▲) $r = 0.75$; incubation temperature 35 °C. The drawn lines were calculated according to the kinetic model for the corresponding off-rate constants given in Table I.

(Figure 3E), a separate observation of both peaks was no longer possible above 80% of equilibration. Thus, we cannot exclude a biexponential behavior of the data in this region.

The calculated off rate with which DMPC molecules escape from a DMPC vesicle in the initial stage of exchange was 20% lower for symmetric exchange (4.5×10^{-5} s^{-1}) than that for asymmetric lipid transfer (5.6×10^{-5} s^{-1}) at 35 °C.

Effect of Incubation Temperature. Changes in incubation temperature produced qualitatively similar effects on both symmetric and asymmetric transfer of phospholipids between two vesicle populations. The effect of the incubation temperature T_{inc} on the transfer kinetics can be inferred from Table I and the insert in Figure 2. As is shown in the insert of Figure 2, the transfer half-time decreases exponentially with increasing T_{inc} (for $T_{inc} > T_c$ of the higher melting lipid component) for the case of asymmetric transfer. These results disagree with those of Martin and MacDonald (1976), who claimed that the transfer kinetics between DMPC and DPPC vesicles does not depend on T_{inc} at $T_{inc} > 50$ °C. At 35 and 28 °C, where DPPC is in the gel state, the lipid transfer is still astonishingly fast, although the log ($t_{1/2}$) vs T_{inc} plot becomes nonlinear. Only incubation temperatures below the T_c of DMPC (23.9 °C) prevented the transfer completely.

This is in striking contrast to the case of symmetric transfer between vesicles of the protonated lipid and those of the deuteriated lipid analogue. In this case transfer was observed only when both vesicles were in the fluid state.

Effect of Vesicle Concentration on Transfer Kinetics. In order to study the effect of lipid concentration on the transfer kinetics, both the total lipid concentration at constant vesicle molar fraction r and r at constant total lipid concentration were varied. The total lipid concentration was varied between 0.2 and 3 mg/mL. No appreciable change of the transfer kinetics could be observed within this concentration range. This finding rules out collisions between vesicles as a mechanism to explain the observed lipid transfer [cf. Roseman and Thompson (1980) and references cited therein].

The variation of r at constant total vesicle concentration had a drastic effect on the kinetics of the asymmetric transfer between DMPC and DPPC vesicles. Figure 5 shows that the transfer rate increases with increasing relative concentration of the DPPC "acceptor" vesicles. This feature is in agreement with results obtained by Duckwitz-Peterlein et al. (1977).

For the symmetric transfer of lipids between DMPC and DMPC-d_{54} vesicles the transfer kinetics did not depend on the vesicle molar fraction r. This is a further characteristic difference between symmetric and asymmetric lipid transfer.

In order to check if the off rate of DMPC molecules depends on the composition of the bilayer from which the monomers escape, the asymmetric transfer was studied between a vesicle population of a equimolar mixture of DMPC/DPPC and an population of pure DPPC vesicles (corresponding to a vesicle molar fraction $r = 0.75$). At $T_{inc} = 35$ °C, the half-time of this transfer was 1.8 h; the off rate of the initial transfer stage was calculated as 1.9×10^{-4} s^{-1}. This is in good agreement with the values obtained for the transfer between pure DMPC and DPPC vesicles at $r = 0.75$ (Table I). Thus, one can conclude that the off rates of DMPC were constant over a large range of bilayer compositions for the case of asymmetric lipid transfer. This is essential for the theoretical treatment of the transfer process in terms of the model of Thilo (1977), which is based on the assumption that the off rate does not depend on the bilayer composition. Therefore, a more sophisticated kinetic model which includes the possibility that monomer association and dissociation vary with the bilayer composition (Nichols & Pagano, 1981) was not considered.

Effect of the Acyl Chain Length on the Transfer Kinetics. In order to elucidate the dependence of lipid transfer on the monomer vesicle dissociation constant, which is expected to depend on the chain length (Tanford, 1973), asymmetric transfer was studied for DLPC–DMPC vesicle mixtures and for mixtures of DPPC vesicles with DSPC vesicles. In addition, symmetric lipid exchange was observed for DPPC vesicles with DPPC-d_{62} vesicles. From the results presented in Table I it follows that $t_{1/2}$ of asymmetric transfer depends drastically on the chain length of the donor vesicles. A closer inspection shows that the transfer half-time $t_{1/2}$ increases exponentially with increasing chain length.

Another interesting result is that the off rate of DPPC dissociated from DPPC vesicles, obtained for the case of symmetric exchange between DPPC and DPPC-d_{62} vesicles, was 20% lower (2.3×10^{-6} s^{-1}) than the value of K^{off} for the asymmetric transfer between DPPC and DSPC vesicles (2.9

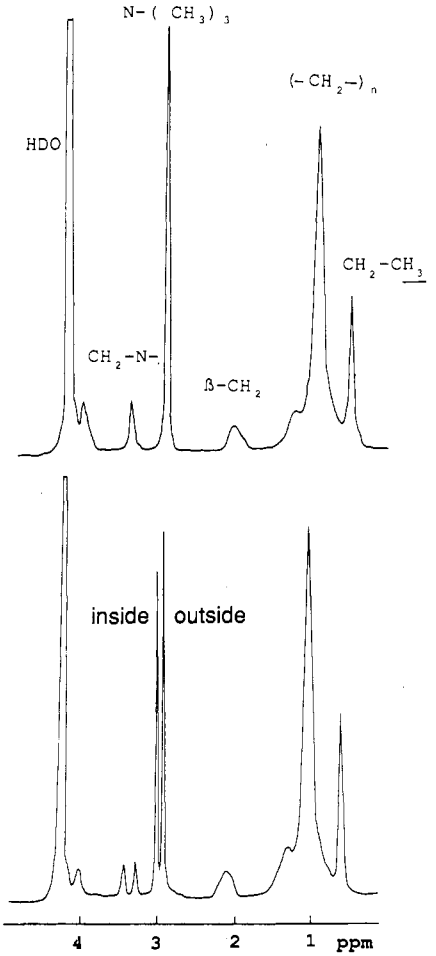

FIGURE 6: 400-MHz ^{1}H NMR spectrum of DPPC vesicles at 45 °C (top spectrum) and on addition of 2 mM $EuCl_3$ (bottom spectrum).

$\times 10^{-6}$ s^{-1}) at identical T_{inc} (55 °C). This reduction is well beyond the range of the experimental uncertainty.

Changes of the Molar Lipid Ratio between Outer and Inner Monolayers of the Vesicles during Transfer. The distribution of the lipids between the outer and the inner monolayer and its changes during lipid transfer were studied by ^{1}H NMR experiments. The outside to inside molar lipid ratio ($R_{o/i}$) was obtained from the splitting of the N-$(CH_3)_3$ group resonance of choline upon addition of a paramagnetic shift reagent (Figure 6). For pure vesicles of DMPC or DPPC (before incubation), a value of $R_{o/i}$ = 1.1 was obtained. This is smaller than expected from our size measurements using DLS and electron microscopy, which yielded a mean external vesicle radius of 19 nm. Assuming that the bilayer thickness of this vesicles is the same as that for multilayers, the mean inner radius should be about 16 nm. Under the assumption of uniform phospholipid packing one expects a value of $R_{o/i}$ = $(19/16)^2$ = 1.4. This discrepancy provides evidence for a different lipid packing density as compared to large unsonicated vesicles.

The changes of $R_{o/i}$ as a function of incubation time after mixing of the two vesicle populations as listed in Table II revealed substantial differences between asymmetric and symmetric lipid transfer. For asymmetric transfer between DMPC and DPPC vesicles, $R_{o/i}$ increased during the initial stage of transfer from 1.1 to 1.5 and slowly decreased later below its initial value to 0.8.

In contrast, the values of $R_{o/i}$ obtained for symmetric exchange increase monotonically from 1.1 to 1.4 during 28 h of incubation time.

Table II: Variation of the Molar Ratio in the Outer Monolayer to That in the Inner Monolayer ($R_{o/i}$) during Incubation

incubation time (h)	$R_{o/i,asym}$[a]	$R_{o/i,sym}$[b]	incubation time (h)	$R_{o/i,asym}$[a]	$R_{o/i,sym}$[b]
0.1	1.1	1.1	6	1.2	
0.6	1.5		7		1.4
1.0		1.2	10	1.1	1.4
1.2	1.5		15	0.9	
2	1.4	1.2	20	0.8	1.4
3.1	1.3	1.3	25	0.8	1.4
5		1.3	28	0.8	1.4

[a] Asymmetric lipid transfer of DMPC to DPPC vesicles (T_{inc} = 45 °C). [b] Symmetric transfer between DMPC and DMPC-d_{54} vesicles (T_{inc} = 35 °C).

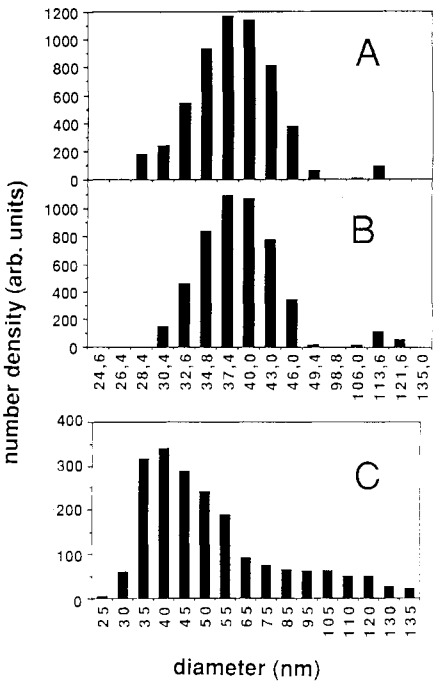

FIGURE 7: Size distribution of an equimolar mixture of DMPC and DPPC vesicles (r = 0.5) at the beginning of incubation at 45 °C determined by DLS (A) and freeze-fracture electron microscopy (C) and after 36 h of incubation at 45 °C determined by DLS (B).

Size Changes of the Vesicles during Lipid Transfer. In order to study changes of the size distribution and the formation of multilamellar structures during the later stages of lipid transfer, the size distribution was measured by DLS and freeze-fracture electron microscopy after various incubation times. Some characteristic results are presented in Figure 7. Clearly, the maxima of the distributions agree well for both methods. The size distribution obtained by DLS, however, was found to be slightly bimodal, while that yielded by electron microscopy always showed an asymmetric shape. It is likely that the small amount of larger vesicles was caused by vesicles which fused during the 8-h equilibration period after sonication and ultracentrifugation. That the electron microscopy data did not exactly confirm a bimodal distribution may be explained by preparative effects such as slight changes of the size distribution during the freezing procedure.

As expected, the average diameter of DMPC vesicles above their T_c was measured to be slightly lower (32 nm) than that of DPPC vesicles (37 nm) by DLS.

As follows from the DLS data presented in Figure 7, the size distribution did not change appreciably during 36 h of incubation at 45 °C. The same invariance of the size distribution was observed for the case of the symmetric exchange. These results were confirmed by freeze-fracture electron

microscopy. Taking into account the accuracy of the size measurements, one can conclude that the average size of the vesicles was not changed more than 4 nm during the incubation period considered.

Discussion

Two limiting cases of lipid transfer between sonicated vesicles were studied in this work. (1) The symmetric exchange of lipids is characteristic for vesicle mixtures of very similar (as DMPC and DMPC-d_{54}) or equal compositions. (2) The asymmetric transfer of lipids between vesicles of different composition generally involves a net transfer of lipids between the different vesicle populations. The former case does not involve a net mass transfer of lipid; i.e., the vesicles exchange lipids in a 1:1 ratio, which should not appreciably affect the vesicle size and structure. In the latter case, one vesicle population is continuously depleted of its lipids until the vesicles become unstable. A mixture of DMPC and DPPC vesicles prepared by detergent dialysis, which yields larger average vesicle diameters (200–900 nm) than sonication, behaves significantly different from sonicated vesicles (Figure 1). As already reported by Papahadjopoulos et al. (1976) for vesicles constituted of charged lipids, the appearance of a new broad central peak can be explained in terms of vesicle fusion. Residual bile salts in the dialysis vesicle preparation are unlikely to be the reason for this behavior. The hs-DSC endotherms are very sensitive for the presence of bile salts and change in a characteristic way at concentrations above 2% (mol) sodium deoxycholate. Moreover, the bile salts accelerate drastically the lipid transfer between vesicles (T. M. Bayerl, unpublished results).

One can conclude that the dominating mechanism by which vesicles exchange lipids (that is, lipid transfer via the bulk solution or vesicle fusion) depends strongly on the size of the vesicles. As lipid transfer is obviously dominating in small sonicated vesicles, all further measurements in this work considered this type of vesicle.

The factors controlling the kinetics of lipid transfer between vesicles can be summarized as follows:

The transfer half-time $t_{1/2}$ also depends exponentially on the incubation temperature for both symmetric and asymmetric lipid transfer. The latter occurs even if the higher melting lipid species is in the gel state, whereas the former requires that both vesicle populations are in the fluid state.

The transfer half-time $t_{1/2}$ depends also exponentially on the acyl chain lengths. This is in good agreement with theoretical predictions. The work associated with the transfer of a lipid monomer between the bilayer and the aqueous solution is proportional to the length of its hydrophobic tail (Tanford, 1973). Consequently, the off rate follows an exponential law. Since the degree of asymmetry of the transfer is determined by the difference between the off rates of the two vesicle populations, it depends exponentially on the chain length, i.e., on the ratio of the monomer concentrations of the lipids. This ratio has been estimated for DMPC and DPPC as $[M_{DMPC}]/[M_{DPPC}] = 35$ by Thilo (1977). From the symmetric exchange results of this work a somewhat lower ratio of $K^{off}_{DMPC}/K^{off}_{DPPC} = 20$ is obtained.

The fact that the calculated off rates for different chain lengths were always about 20% lower for symmetric transfer than for the asymmetric one suggests that the latter is accelerated by additional forces (see below).

Our finding that the lipid-transfer kinetics was independent of the total vesicle concentration is in agreement with the papers cited in the introduction except for that of Kremer et al. (1977). These authors studied vesicles prepared by the ethanol injection method, which are expected to be much larger than sonicated vesicles. Therefore, as suggested by our results for dialysis vesicles, it is likely that collision-dependent vesicle fusion rather than lipid transfer was dominating in their experiments.

The dependence of the transfer kinetics on the vesicle molar fraction r at constant total vesicle concentration in the case of asymmetric lipid transfer, which was not observed for symmetric transfer is caused by the fact that the former always involves a net transfer of lipids between the vesicles (see below).

The observed size invariance of the vesicles during both types of transfer excludes an appreciable shrinking of one vesicle population due to lipid depletion by asymmetric transfer.

One of the most interesting features is the splitting of the hs-DSC endotherms in the initial stage of asymmetric lipid transfer. This initial splitting of the DPPC transition peak (Figure 1A, trace b) is caused by an imbalance in distribution of DMPC between the inner and the outer monolayer of the DPPC acceptor vesicles. As reported by deKruijff and Wirtz (1977), the equilibration of lipids between both monolayers by flip-flop in unstained phosphatidylcholine vesicles is a very slow process with an estimated half-time of days. As the off rate of DPPC is an order of magnitude smaller than that of DMPC (Table I), the outer monolayer accepts more monomeric lipids than can be transported to the inner layer by flip-flop or released to the bulk solution. This can be accomplished by a tighter packing of the lipids in the outer monolayer and/or by insertion of lipids into vacancies and defects. Consider the situation in Figure 1A, trace b: The maximum splitting of 2.6 °C allows an estimation of the excess DMPC that can be accepted by the outer DPPC monolayer. Using eq 1 and taking into account the net mass ratio $R_{o/i} = 1.1$ as determined by ^{1}H NMR for pure DPPC vesicles, one obtains a 30% increase of lipid constituents in the outer monolayer, corresponding to an increase of $R_{o/i}$ of about 0.3. This is in very good agreement with the value of $R_{o/i} = 1.5$ determined for this mixture at 0.6 h incubation time by ^{1}H NMR (Table II).

The gradual decrease in intensity and shift toward lower temperatures of the sharp part of the split DPPC peak, the subsequent coalescence of the two peaks after 50 min of incubation (Figure 1A, traces c,d), and the final temperature shift of the coalesced peak toward the equilibrium position (Figure 1 A, trace e) give evidence for a driven flip-flop between outer and inner monolayer. The reason for this increase of the flip-flop rate of about 1 order of magnitude could be explained in terms of the increased lateral pressure in the outer monolayer.

Additionally, an osmotic force can accelerate this process, for the monomer lipid concentration in the outer aqueous solution is at least a factor of 10 higher than in the inner aqueous space of the acceptor vesicles owing to the different off rates for DPPC and DMPC (Table I). This can cause a loss of water from the inner space and/or an increased transport of DMPC monomers to the inner monolayer. The above-mentioned 20% difference between the off rates of DMPC and DPPC obtained from symmetric and asymmetric transfer (cf. Table I) may reflect the presence of an additional force such as the osmotic one, which is not present in symmetric lipid transfer, for the off rates of DMPC and DMPC-d_{54} are equal. This additional force can also explain our finding that the asymmetric transfer occurred even while the higher melting lipid was still in the gel state. The observed acceleration of the asymmetric transfer kinetics with increasing vesicle molar fraction r (Figure 5) then simply reflects the presence of more acceptor (DPPC) vesicles. This results in

a higher molar fraction of DMPC molecules incorporated in DPPC vesicles before the flip-flop rate is forced to increase. Such a dependence on r is not expected in symmetric transfer experiments, where each vesicle is simultaneously donor and acceptor. This is in agreement with the observed first-order kinetics.

The time course of the transfer calculated for a constant lipid off rate following Thilo (1977) (cf. Figures 2, 4, and 5), fits well with the initial stage of the asymmetric lipid transfer (Figures 2 and 5) and for the whole course of transfer in the case of symmetric lipid transfer (Figure 4). This supports the validity of one of the basic assumptions of this model, that the off rate of the lipids is the rate-determining step for the initial stage of asymmetric transfer and for large regions of symmetric lipid transfer. Changes of the off rates with variations of the bilayer composition are unlikely to be the reason for the biexponential behavior of the kinetics in asymmetric lipid transfer, as the off rate of DMPC was found to be constant over a large region of bilayer compositions (see Results).

For the case of asymmetric transfer, the deviations from the single-exponential course of the Thilo model for longer incubation times (Figure 2) can be explained as follows:

(1) After saturation of the outer monolayer of the acceptor vesicles, the flip-flop rate increases. This might depend on the chemical potential difference between the outer and the inner monolayer and the osmotic gradient between the outer and the inner aqueous phase of the acceptor vesicles. As the capacity of the outer monolayer to accept excess DMPC is restricted (30% as discussed above), the on rate of DMPC to the acceptor vesicle will drop until it becomes equal to the increased flip-flop rate. Thus, the equilibration between the two monolayers by flip-flop now becomes the rate-determining step.

(2) The DMPC donor vesicles are depleted of their lipid constituents during transfer but do not change their size appreciably. Lipid depletion without shrinking could be possible by increasing the motional freedom of the remaining lipids. Reaching a critical degree of depletion the vesicles become unstable and possibly fuse with other unstable vesicles. This process too can slow down the transfer kinetics in the final stage of asymmetric transfer.

The experimental finding of a monolayer mass ratio of the composite vesicles of $R_{o/i} = 0.8$ at the final stage of asymmetric lipid transfer is an interesting point. Since vesicles with such a mass imbalance in favor of the inner monolayer can hardly be stable, we conclude that vesicles with more than one bilayer in the average emerge from this process. This might explain the observed size invariance of the vesicles. The freeze-fracture micrographs, however, gave no evidence for the presence of typical multilamellar vesicles at the final transfer stage. Since no splitting of the transition endotherms in the initial stage of symmetric lipid transfer was observed (Figure 3), we conclude that lipid flip-flop is always fast enough to equilibrate imbalances of lipid composition between the two monolayers, which implies that the off rate of DMPC and DMPC-d_{54} is lower than the flip-flop rate between the monolayers of the vesicles. This situation might change in the later stages, as is indicated by the occurrence of a splitting of the heat capacity curves (Figure 3C). The splitting correlates with the slight increase of $R_{o/i}$ without size changes during symmetric exchange from 1.1 up to the theoretically expected value of 1.4. This can be understood in terms of a slow self-annealing of defects and vacancies in the outer monolayer by lipid transfer.

ACKNOWLEDGMENTS

We thank Irene Sprenger for help in electron microscopy, Angelika Kurrle for her assistance in preparation of dialysis vesicles, and Sibylle Schardey for help in preparation of the manuscript. T.M.B. is grateful to Prof. Thomas E. Thompson for useful discussions.

Registry No. DLPC, 18194-25-7; DMPC, 18194-24-6; DPPC, 63-89-8; DSPC, 816-94-4.

REFERENCES

DeKruijff, B., & Wirtz, K. W. A. (1977) *Biochim. Biophys. Acta 468*, 318.

Duckwitz-Peterlein, G., Eilenberger, G., & Overath, P. (1977) *Biochim. Biophys. Acta 469*, 311.

Enoch, H. G., & Strittmatter, P. (1979) *Proc. Natl. Acad. Sci. U.S.A. 77*, 145–146.

Kremer, J. M. H., Kops-Werkhoven, M. M., Pathmamanoharan, O. L. J., Gijzeman, O. L. J., & Wiersema, P. H. (1977) *Biochim. Biophys. Acta 471*, 177.

Lentz, B. R., Carpenter, T. J., & Alford, D. R. (1987) *Biochemistry 26*, 5389.

Martin, F. J., & MacDonald, R. C. (1976) *Biochemistry 15*, 321.

McLean, L. R., & Phillips, M. C. (1981) *Biochemistry 20*, 2893.

Nichols, J. W., & Pagano, R. E. (1981) *Biochemistry 20*, 2783.

Nichols, J. W., & Pagano, R. E. (1982) *Biochemistry 21*, 1720.

Papahadjopoulos, D., Hui, S., Vail, W. J., & Poste, G. (1976) *Biochim. Biophys. Acta 448*, 245.

Patton, G. M., Robins, S. J., Fasulo, J. M., & Clark, S. B. (1985) *J. Lipid Res. 26*, 1285.

Provencher, S. W. (1976) *Biophys. J. 16*, 27.

Provencher, S. W. (1982a) *Comput. Phys. Commun. 27*, 213.

Provencher, S. W. (1982b) *Comput. Phys. Commun. 27*, 229.

Provencher, S. W., & Vogel, R. H. (1980) *Math. Biosci. 50*, 251.

Roseman, M. A., & Thompson, T. E. (1980) *Biochemistry 19*, 439.

Silvius, J. R., Leventis, R., Brown, P. M., & Zuckermann, M. (1987) *Biochemistry 26*, 4279.

Tanford, C. (1973) *The Hydrophobic Effect*, Wiley, New York.

Thilo, L. (1977) *Biochim. Biophys. Acta 469*, 326.

Xü, Y. H., Gietzen, K., Galla, H.-J., & Sackmann, E. (1983) *Biochem. J. 209*, 257.