Schiffer, M., Hardman, K. D., Wood, M. K., Edmundson, A. B., Hook, M. E., Ely, K. R., & Deutsch, H. F. (1970) *J. Biol. Chem. 245*, 728.
Schiffer, M., Girling, R. L., Ely, K. R., & Edmundson, A. B. (1973) *Biochemistry 12*, 4620.
Segal, D. M., Padlan, E. A., Cohen, G. H., Rudikoff, S., Potter, M., & Davies, D. R. (1974) *Proc. Natl. Acad. Sci. U.S.A. 71*, 4298.
Silverton, E. W., Navia, M. A., & Davies, D. R. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 5140.
Wang, B.-C., Yoo, C. S., & Sax, M. (1979) *J. Mol. Biol. 129*, 657.
Zeppezauer, M., Ecklund, H., & Zeppezauer, E. S. (1968) *Arch. Biochem. Biophys. 126*, 564.
Zidovetzki, R., Licht, A., & Pecht, I. (1979) *Proc. Natl. Acad. Sci. U.S.A.* (in press).

Mechanism of the Spontaneous Transfer of Phospholipids between Bilayers†

M. A. Roseman‡ and T. E. Thompson*

ABSTRACT: A fluorescent phospholipid, 1-palmitoyl-2-pyrenedecanoylphosphatidylcholine, was used to study the mechanism of spontaneous phospholipid transfer between single-walled phospholipid vesicles. The half-time for transfer of this molecule between vesicles of dimyristoylphosphatidylcholine at 36 °C is 13 h if flip-flop is negligible or 24 h if flip-flop is faster than intervesicle exchange. The half-time is unaffected by the concentration of acceptor vesicles, which indicates that transfer of label takes place by diffusion of monomers or micelles through the aqueous phase rather than by collision of vesicles. These results are compared with previous studies of spontaneous lipid transfer.

Recent studies have shown that phospholipids spontaneously transfer between phospholipid vesicles with half-times that range from 2 to 24 h, depending on the particular lipid and temperature (Martin & MacDonald, 1976; Duckwitz-Peterlein et al., 1977; Kremer et al., 1977; Papahadjopoulos et al., 1976).[1] In contrast to the obligatory one-for-one exchange reaction catalyzed by phospholipid exchange proteins (Wirtz, 1974), spontaneous flux usually leads to a net transfer of lipid from one membrane to another. This process, if it occurs in vivo, may in part determine the composition of biological membranes.

The purpose of the present study is to determine the mechanism of spontaneous phospholipid transfer. Presumably, there are only two simple mechanisms, which are kinetically distinguishable: either (1) lipid transfers as monomers or micelles through the aqueous phase or (2) lipid transfers upon collision of the membranes. In the studies cited above, calorimetric or light-scattering techniques were used to measure the transfer of phospholipids between liposomes or unilamellar vesicles composed of phospholipids having different gel–liquid-crystal phase transition temperatures. From their results, Papahadjopoulos et al. (1976), Martin & MacDonald (1976), and Duckwitz-Peterlein et al. (1977) support the first mechanism whereas Kremer et al. (1977) support the second.

We have used a different approach to measure transfer, based on the methods of Doody et al. (1978), Charlton et al. (1976, 1978), and Sengupta et al. (1976), which employ pyrene-labeled lipids. In our case, we have measured the exchange kinetics of 1-palmitoyl-2-pyrenedecanoylphosphatidylcholine (pyrene-PC[2]) between single-walled vesicles of dimyristoylphosphatidylcholine. Our results clearly show that transfer of label takes place through the aqueous phase rather than by vesicle collision. The half-time at 36 °C is 13 h if equilibration of label across bilayers (flip-flop) is negligible; if flip-flop is faster than exchange between vesicles, the half-time is 24 h. A preliminary report of our studies has appeared elsewhere (Roseman & Thompson, 1979). Recently, Galla et al. (1979), using a virtually identical approach, obtained completely different results; they reported that most of the pyrene-PC transfers between dimyristoylphosphatidylcholine and dipalmitoylphosphatidylcholine vesicles with a half-time of ~11 s (23 °C), followed by slow transfer of the remaining probe with a half-time of 8 h (50 °C). The result obtained by Galla et al. (1979) is also in marked disagreement with the results obtained by Martin & MacDonald (1976), Papahadjopoulos et al. (1976), Duckwitz-Peterlein et al. (1977), and Kremer et al. (1977).

Materials and Methods

Dimyristoyl- and dipalmitoylphosphatidylcholines, purchased from Sigma, were purified further by silicic acid column chromatography, acetone precipitation, and extraction with chloroform–methanol–H_2O (8:4:3) containing EDTA. Pyrenedecanoic acid was purchased from Molecular Probes.

1-Palmitoyl-2-pyrenedecanoylphosphatidylcholine was prepared from dipalmitoylphosphatidylcholine according to the method used by Roseman et al. (1978) to prepare 1-palmitoyl-2-oleylphosphatidylcholine. This method, which employs fatty acid anhydrides, contains minor modifications of the procedures developed by Cubero Robles & van den Berg (1969). The absorption and fluorescence spectra of pyrene-PC were identical with those of pyrenedecanoic acid. Galla et al. (1979) found the critical micelle concentration of pyrene-PC to be 10^{-7} M. This is close to the value of 3×10^{-8} M calculated for dimyristoylphosphatidylcholine by Martin &

† From the Department of Biochemistry, University of Virginia School of Medicine, Charlottesville, Virginia 22908. *Received August 9, 1979.* This work was supported by U.S. Public Health Service Grants GM-14628 and GM-23573.
‡ Present address: Department of Biochemistry, Uniformed Services University of Health Sciences, Bethesda, MD 20014.

[1] These references do not include the large number of studies carried out with phospholipid exchange protein on biological membranes, in which the slow uncatalyzed, spontaneous isotopic equilibration of lipids is routinely measured as a control (Shaw et al., 1979).
[2] Abbreviations used: pyrene-PC, 1-palmitoyl-2-pyrenedecanoylphosphatidylcholine.

0006-2960/80/0419-0439$01.00/0

MacDonald (1976) and close to the value determined by Thilo (1977) for a similar phosphatidylcholine.

Dispersions of homogeneous single-walled vesicles were prepared, in 0.05 M KCl–0.01 M Tris-HCl–10^{-4} M EDTA (pH 7.5), by the method of Barenholz et al. (1977). Dimyristoylphosphatidylcholine vesicles containing 1–4 mol % pyrene-PC were prepared from an initial solution of the two lipids in chloroform. The temperature of all the vesicle preparations was never permitted to drop below 30 °C. Vesicles were incubated for 12 h at 37 °C before use. Phospholipid phosphorus was determined by the method of Bartlett (1959).

Fluorescence Measurements. The principle underlying the pyrene-PC transfer experiment is as follows (Doody et al., 1978; Charlton et al., 1976, 1978; Sengupta et al., 1976). When a pyrene molecule in the excited state collides with a pyrene molecule in the ground state, a short-lived excited complex called an eximer is formed which emits light at much longer wavelength than does the monomer. Since eximer formation is a bimolecular reaction, dependent on the concentration of probe in the membrane, the ratio of eximer to monomer fluorescence falls during net transfer of probe from labeled to unlabeled vesicles. By following the change in this ratio, the kinetics and thus the mechanism of transfer can be obtained.

If a fixed number of probes are evenly distributed among all the vesicles (and within each vesicle), the ratio of eximer to monomer fluorescence intensity (E/M) is given by the expression

$$\frac{E}{M} = \frac{C}{C_h}\frac{E_{max}}{M_{max}} \quad (1)$$

Here C is the number of probes per phospholipid molecule, E_{max} is the eximer intensity that would be observed as the number of phospholipid molecules approaches zero, i.e., as $C \rightarrow \infty$, M_{max} is the monomer intensity observed as the number of phospholipid molecules approaches inifinity, i.e., as $C \rightarrow 0$, and C_h is the half-value concentration. By measuring E/M in a series of vesicle preparations containing increasing concentrations of probe, the constants C_h and E_{max}/M_{max} can be determined. These constants are needed to determine properly the kinetics of probe transfer between vesicle populations because the observed values of E/M are not related in a simple way to the extent of probe transferred (except at $t = 0$ and $t = \infty$); at any time during transfer, the observed value of E/M is given by

$$\frac{E_{obsd}}{M_{obsd}} = \frac{E_D + E_A}{M_D + M_A} \quad (2)$$

Here the subscripts D and A refer to donor and acceptor vesicles. The ultimate expression relating E_{obsd}/M_{obsd} to the concentration of probe in the donor vesicles, C_D, is

$$\frac{E_{obsd}}{M_{obsd}} = \frac{C_D^2(C_{D_0} - C_D) + RC_D^2C_h + (C_{D_0} - C_D)^2(C_D + C_h)}{C_hC_D(C_{D_0} - C_D) + RC_h^2C_D + RC_h(C_{D_0} - C_D)(C_D + C_h)}\cdot\frac{E_{max}}{M_{max}} \quad (3)$$

Here C_{D_0} is the concentration in the donor vesicles at $t = 0$ and R is the ratio of acceptor to donor vesicles. The derivation of eq 3 is given in the Appendix.

Fluorescence measurements were made at 36 °C with a Perkin-Elmer MPF-3 spectrofluorometer, using uncorrected spectra. Monomer and eximer emission were measured at 376 and 476 nm, respectively, using an excitation wavelength of 320 nm. The eximer/monomer fluorescence intensity ratio of dimyristoylphosphatidylcholine vesicles containing 0–4 mol % pyrene-PC was linear with respect to probe concentration. No attempt was made to exclude oxygen because oxygen quenching does not affect the kinetics. Under these conditions, the excitation and emission spectra of pyrene-PC-containing vesicles in the absence of acceptor vesicles remained unchanged over the time period of the experiments.

Table 1: Eximer/Monomer Intensity Ratios (E/M) of Dimyristoylphosphatidylcholine Vesicles Containing 3.8 mol % Pyrene-PC in the Presence and Absence of Unlabeled Dimyristoylphosphatidylcholine Vesicles

	E/M[a]			
time (h)	$R = 0$	$R = 5$	$R = 10$	$R = 20$
0.167	0.432	0.417	0.415	0.409
0.417	0.438	0.414	0.409	0.406
0.917	0.437	0.399	0.391	0.384
1.42	0.442	0.391	0.380	0.373
1.83	0.442	0.379	0.368	0.361
2.83	0.444	0.353	0.345	0.335
3.83	0.446	0.334	0.324	0.317
4.83	0.446	0.320	0.306	0.297
5.83	0.441	0.302	0.285	0.276
6.83	0.434	0.295	0.275	0.265
7.83	0.432	0.274	0.258	0.248
8.83	0.446	0.262	0.243	0.231
10.18	0.443	0.249	0.230	0.219
12.08	0.428	0.228	0.213	0.201

[a] R = ratio of unlabeled to labeled vesicles at $t = 0$.

The lipid transfer experiments were initiated as follows. Into four cuvettes was added an identical amount of dimyristoylphosphatidylcholine (donor) vesicles containing 3.8 mol % pyrene-PC, so that the final concentration of labeled vesicles would be 0.13 mM in lipid phosphorus. After initial measurements were taken, unlabeled dimyristoylphosphatidylcholine (acceptor) vesicles were added to three cuvettes so that the final ratios of acceptor to donor vesicles were 5, 10, and 20. The fourth solution, containing only donor vesicles, served as a control. Light scattering was negligible.

Results and Discussion

As shown in Table I, when dimyristoylphosphatidylcholine vesicles containing 3.8 mol % pyrene-PC were incubated with a 5-, 10-, or 20-fold excess of dimyristoylphosphatidylcholine vesicles at 36 °C, the eximer/monomer fluorescence intensity ratio dropped slowly and continuously, reaching a half-equilibrium value in all cases, after ~12 h. Three processes could account for the decrease in this ratio: (1) net transfer of pyrene-PC from labeled to unlabeled vesicles, (2) net transfer of dimyristoylphosphatidylcholine from unlabeled to labeled vesicles driven by the small dimyristoylphosphatidylcholine concentration gradient, (3) fusion of labeled and unlabeled vesicles.

If either the second or third process is important, the average molecular weight of the vesicles should have greatly increased. This was ruled out in a separate experiment using the spectrofluorometer to monitor the 90° light scattering of a mixture containing unlabeled/labeled vesicles in a ratio of 2:1. After 12 h, when the eximer/monomer intensity ratio had reached the half-equilibrium value, the increase in light scattered at

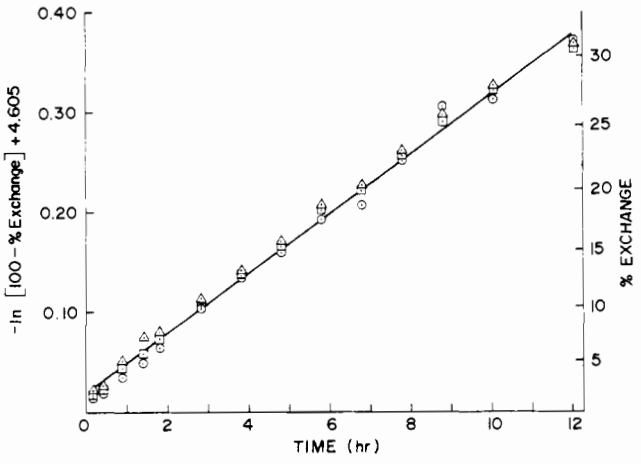

FIGURE 1: Kinetics of pyrene-PC transfer between dimyristoylphosphatidylcholine vesicles at 36 °C. The ratios of acceptor to donor vesicles in the three reaction mixtures are 5 (⊙), 10 (□), and 20 (△). For this plot, the values of percent exchange are based on the assumption that all the pyrene-PC molecules transfer as a single kinetic pool.

420 nm was no greater than 7%. Since small unilamellar vesicles are Rayleigh scatterers (Barenholz et al., 1977), if the loss of eximer to monomer fluorescence had resulted from net dimyristoylphosphatidylcholine transfer or vesicle fusion, the intensity of light scattered by the 2:1 vesicle mixture should have increased by at least 67%. Thus, the process must be net transfer of pyrene-PC. A control experiment showed that the light scattering from dimyristoylphosphatidylcholine vesicles alone increased by 7% over this period and then continued to increase ever more rapidly; at 21 h, the increase was 25%. Of course, these increases in light scattering could have resulted from aggregation of the particles. Because of these uncertainties, however, no results beyond 12 h were considered reliable. Nevertheless, the eximer/monomer ratios did approach the expected equilibrium values after several days.

Equation 1 was used to calculate the mole fraction of label remaining in the donor vesicles as a function of time, from which the kinetics of transfer was obtained. These results, plotted as a reversible first-order process, are shown in Figure 1. Clearly, the half-time for label transfer does not depend on the concentration of acceptor vesicles. For the following reasons, this result is consistent only with the mechanism wherein lipid monomers or micelles exchange between bilayers through the aqueous phase rather than by collision of the vesicles. If vesicle collisions are required, the rate of transfer must be proportional to the product of the concentration of donors and acceptors. But if exchange proceeds via transfer of lipid monomers through the aqueous phase, the half-time of transfer will be independent of the acceptor vesicle concentration because the rate-determining step must be dissociation of the monomer from the donor vesicle. This follows from the fact that the monomer–vesicle equilbrium dissociation constant, which is the ratio of the "off" rate constant to the "on" rate constant, is exceedingly small. More detailed treatments of these kinetics have been presented by Charlton et al. (1978), Lawaczeck (1978), Thilo (1977), and Duckwitz-Peterlein & Moraal (1978).

Since meaningful data for pyrene-PC transfer could not be obtained beyond 12 h, it is not known whether all or only a portion of the label transfers according to the single-exponential decay process shown in Figure 1. The plot shown in Figure 1 is obtained if it is assumed that equilibration of pyrene-PC across the bilayer (flip-flop) is much faster than interbilayer transfer. This gives a half-time of ~24 h for label transfer.

If flip-flop of label is assumed to be negligible over the 12-h period of the measurements, plots nearly identical in appearance with that of Figure 1 are obtained, but with larger slopes. In order to calculate the half-time for label transfer under these circumstances, it is necessary to know the outside/inside monolayer mass ratio for dimyristoylphosphatidylcholine vesicles. The two groups that have studied dimyristoylphosphatidylcholine vesicle structure in detail have obtained somewhat conflicting results (Aune et al., 1977; Watts et al., 1978). For the limiting case calculations, we have chosen to use values obtained near 36 °C that correspond to vesicles with the smallest outside/inside monolayer mass ratio, because vesicles of such structure should have the largest nonexchanging pool of pyrene-PC. The values obtained at 30 °C by Watts et al. (1978) appear to fulfill this requirement; if the external radius is 112 Å and the internal radius is 82 Å, the monolayer mass ratio should be approximately $(112/82)^2 = 1.86$, assuming uniform packing of the lipids. By use of this number to calculate the end point for pyrene-PC transfer, a lower limit of 13 h is obtained for the half-time.

As indicated earlier, other laboratories have studied lipid transfer by calorimetric and light-scattering techniques (Martin & MacDonald, 1976; Duckwitz-Peterlein et al., 1977; Kremer et al., 1977; Papahadjopoulos et al., 1976) using lipids with different gel–liquid-crystalline phase transition temperatures. The primary advantage of this technique is that phospholipids having structures typical of those found in biological membranes can be studied. In our studies, we must be concerned with the possibility that pyrene-PC does not behave like a typical phospholipid. Although this possibility cannot be ruled out entirely, two of our results are reassuring. First, the linearity of the eximer/monomer intensity ratio with probe concentration shows that pyrene-PC mixes randomly with dimyristoylphosphatidylcholine rather than forming separate clusters or domains within the vesicle bilayer. Second, the long half-time (13–24 h) for spontaneous transfer of pyrene-PC is compatible with the values found for the natural phospholipids (Martin & MacDonald, 1976; Duckwitz-Peterlein et al., 1977; Kremer et al., 1977; Papahadjopoulos et al., 1976). Of these, dimyristoylphosphatidylcholine shows the fastest exchange rates; isotopic equilibration between single-walled vesicles has a half-time (extrapolated to 37 °C) of ~80 min (DeKruijff & van Zoelin, 1978) whereas net transfer of dimyristoylphosphatidylcholine to dipalmitoylphosphatidylcholine vesicles has a half-time of ~5 h (Martin & MacDonald, 1976; Kremer et al., 1977).

Our results, as well as those cited above, are difficult to reconcile with those of Galla et al. (1979), who reported that pyrene-PC transfers between dimyristoylphosphatidylcholine vesicles with a half-time of 11 s at 23 °C. It is clear from our primary data, shown in Table I, that we observe no rapid phase decrease in eximer to monomer intensity. It is possible that the stopped-flow apparatus employed by Galla et al. (1979) is responsible for an artifact; perhaps the pressure transient or shearing forces incurred upon solution mixing destablize the vesicles and lead to rapid lipid mixing or vesicle–vesicle fusion. It is also possible that the anomalously fast exchange rate observed by Galla et al. (1979) may be caused by the presence of larger liposomes in their unfractionated dispersions or by their failure to anneal the vesicles above the phase transition before use.

In order to interpret correctly the apparent intervesicle lipid transfer kinetics, it is necessary to consider the possible effects

of a transbilayer mass imbalance created by this process. In the calorimetric experiments, a large fraction of the lipid must be transferred before changes in the heat capacity curves are detected. In the studies cited above (Martin & MacDonald, 1976; Papahadjopoulos et al., 1976; Duckwitz-Peterlein et al., 1977; Kremer et al., 1977) the first reliable measurements of transfer are obtained when the acceptor vesicles have increased in mass by at least 10%. At that point, and at all subsequent time points, the heat capacity curves show that the two lipids are randomly mixed in the acceptor vesicle population (Martin & MacDonald, 1976; Duckwitz-Peterlein et al., 1977). Since this can only happen if lipid crosses the bilayer, it is clear that two sequential processes are being measured in these experiments: transfer of lipid between bilayers followed by transfer of lipid across bilayers. The critical question is which of these two steps is rate determining in the overall transfer process. Without this piece of information, it is impossible to extract from the kinetics of net transfer the mechanism of intervesicle lipid transfer.

Since transbilayer migration of phospholipids is normally very slow in vesicles, with half-times ranging from days to weeks (Thompson, 1978), it can be assumed that net lipid transfer between vesicles drives this process. This seems reasonable because as lipid flows from the outer monolayer of donor vesicles to the outer monolayer of acceptor vesicles, mass imbalances must be generated across each bilayer. As the mass imbalances grow, the bilayers become strained and the kinetic barrier to transbilayer equilibration of the lipids is lowered. If this picture is basically correct, transbilayer equilibration of the lipids cannot be faster than interbilayer transfer. However, it may be slower and limit the overall rate of the process if the strained vesicles must reorganize to some extent before accepting or donating more lipid. An additional problem arises when sonicated vesicles are used as donors since these vesicles cannot become much smaller in size. When these vesicles are depleted of lipid to the threshold necessary for vesicle formation, they must either burst or fuse with other vesicles. These processes might also limit the overall rate of lipid transfer.

In our studies with pyrene-PC the mass imbalance effect is either minimal or nonexistent for two reasons. First, pyrene-PC is present at low mole fractions. We are able to obtain meaningful kinetics after 2 h, at which time the fraction of lipid mass lost from the donor vesicles does not exceed 0.4% of the total and the fraction of lipid mass gained by the acceptor vesicles does not exceed 0.07%. Second, the reverse transfer of dimyristoylphosphatidylcholine should readily compensate for even these small changes because dimyristoylphosphatidylcholine is known to transfer much faster than pyrene-PC (Martin & MacDonald, 1976; Kremer et al., 1977; DeKruijff & van Zoelin, 1978). Therefore, in our studies the rate-limiting step must be the transfer of label between membranes.

Among the calorimetric and light-scattering studies cited, only Duckwitz-Peterlein et al. (1977) and Kremer et al. (1977) directly tested the proposed mechanisms for phospholipid transfer by measuring the kinetics of transfer as a function of vesicle concentration. The results obtained by Duckwitz-Peterlein et al. (1977), showing that the half-time for lipid transfer is independent of vesicle concentration, are in complete agreement with our own. However, Kremer et al. (1977) obtained bimolecular kinetics and concluded that exchange requires vesicle collisions. Of greater importance, however, is their finding that in mixtures containing dimyristoylphosphatidylcholine and dipalmitoylphosphatidylcholine vesicles, both lipids transfer at equal rates. This result is completely different than those obtained in the other studies which all show that the relative rates of phospholipid transfer strongly depend on the relative length of the fatty acyl chains. For example, Martin & MacDonald (1976) found that in mixtures of dimyristoylphosphatidylcholine and dipalmitoylphosphatidylcholine vesicles, lipid mixing occurs exclusively by transfer of dimyristoylphosphatidylcholine into dipalmitoylphosphatidylcholine vesicles. Clearly, Kremer et al. (1977) studied a very different process than that observed in other laboratories.

Moreover, the mechanisms proposed for the different exchange processes seem perfectly reasonable. To explain asymmetric lipid transfer between vesicles, it is easiest to envision exchange taking place by transfer of monomers through the aqueous phase. By this mechanism, the rate of transfer should vary according to the monomer–vesicle dissociation constant of each lipid, which in turn varies inversely with the length of the fatty acyl chains. A detailed treatment of this mechanism has been given by Thilo (1977). To explain equal transfer rates, it is easiest to envision the formation of a short-lived vesicle–vesicle collisional complex that destabilizes both bilayers and leads to randomization of the lipids at the region of contact.

The primary difficulty, however, is to find an explanation for the conflicting results obtained by the different laboratories. The only obvious difference in the experimental design used by Kremer et al. (1977) is the use of vesicles prepared by ethanol injection (Batzri & Korn, 1973; Kremer et al., 1977) rather than by sonication. Thus, all of their incubation mixtures contained 0.5–5% ethanol. Although they report that their results are unaffected by ethanol over this concentration range, this does not eliminate the possibility that small quantities of ethanol affect the bilayer structure and cause a collisional transfer mechanism to dominate.

Although it has been known for some time that spontaneous exchange of phospholipid occurs between biological membranes and acceptor vesicles, the details of the process have not been examined. If this exchange, which is not mediated by exchange proteins, is in fact a net mass transfer via aqueous-phase monomers or micelles, then the process could play a role in membrane biogenesis. For example, it seems clear that in the case of mitochondrial biogenesis, it is critical that a net mass transfer of lipid from the site of biosynthesis in the endoplasmic reticulum to the growing mitochondrion take place. It may be that this net transfer is in fact a spontaneous process similar to that observed for pyrene-PC.

Appendix

The expression relating the experimental eximer/monomer intensity ratio to the concentration of probe remaining in donor vesicles is derived as follows.

By use of Förster's theory (Förster, 1969), the quantum yields for eximer emission, η_E, and monomer emission, η_M, are given by

$$\eta_E = \frac{C}{C + C_h}\eta_{E_{max}} \tag{4a}$$

and

$$\eta_M = \frac{C_h}{C + C_h}\eta_{M_{max}} \tag{4b}$$

Here C is the mole ratio of probe to phospholipid, C_h is the half-value concentration, $\eta_{E_{max}}$ is the maximum eximer quantum yield when $C \rightarrow \infty$, and $\eta_{M_{max}}$ is the maximum quantum yield for monomer when $C \rightarrow 0$.

In an aqueous solution of vesicles, the fluorescence intensity of eximer and monomer emission is given by

$$E = \eta_E K_1 CL \tag{5a}$$

and

$$M = \eta_M K_2 CL \tag{5b}$$

Here K_1 and K_2 are proportionality constants and L is the molar concentration of phospholipid. Substituting for η_E and η_M, we obtain

$$E = \frac{C^2 L K_1 \eta_{E_{max}}}{C + C_h} \tag{6a}$$

and

$$M = \frac{C_h C L K_2 \eta_{M_{max}}}{C + C_h} \tag{6b}$$

During lipid transfer, the observed eximer to monomer emission is

$$\frac{E}{M} = \frac{E_D + E_A}{M_D + M_A} \tag{7}$$

where the subscripts D and A refer to donor and acceptor vesicles. Substituting for eq 7

$$\frac{E}{M} = \frac{\left(\frac{C_D^2 L_D}{C_D + C_h} + \frac{C_A^2 L_A}{C_A + C_h}\right)}{\left(\frac{C_h C_D L_D}{C_D + C_h} + \frac{C_h C_A L_A}{C_A + C_h}\right)} \frac{K_1}{K_2} \frac{\eta_{E_{max}}}{\eta_{M_{max}}} \tag{8}$$

Employing a conservation of mass equation, we find that

$$C_A = \frac{1}{R}(C_{D_0} - C_D) \tag{9}$$

Here R is the ratio of acceptor to donor vesicles and C_{D_0} is the concentration of probe in donor vesicles at $t = 0$.

Substituting for C_A and rearranging terms gives

$$\frac{E}{M} = \frac{C_D^2(C_{D_0} - C_D) + RC_D^2 C_h + (C_{D_0} - C_D)^2(C_D + C_h)}{C_h C_D(C_{D_0} - C_D) + RC_h^2 C_D + RC_h(C_{D_0} - C_D)(C_D + C_h)} \cdot \frac{K_1}{K_2} \frac{\eta_{E_{max}}}{\eta_{M_{max}}} \tag{10}$$

The constant C_h was determined as follows from the monomer intensity values of a series of vesicles containing 1–4 mol % pyrene-PC. Equation 6b can be rearranged to

$$\frac{C}{M} = \frac{C^2}{LK_2 C_h \eta_{M_{max}}} + \frac{1}{LK_2 \eta_{M_{max}}} \tag{11}$$

from which a plot of C/M vs. C^2 gives $1/(LK_2\eta_{M_{max}}) \times 1/C_h$ as the slope and $1/(LK_2\eta_{M_{max}})$ as the intercept. Then C_h = 1/[(intercept)(slope)]. In our case, a value of $C_h = 0.102$ was obtained.

Finally, the ratio of constants $K_1\eta_{E_{max}}/(K_2\eta_{M_{max}})$ in eq 10 was experimentally determined from the value of E/M when $C_{D_0} = C_D$. In this limiting case, eq 10 becomes

$$\frac{E}{M} = \frac{C_D}{C_h} \frac{K_1 \eta_{E_{max}}}{K_2 \eta_{M_{max}}} \tag{12}$$

This ratio of constants is equivalent to E_{max}/M_{max} as shown below.

From eq 4a and 4b

$$\frac{\eta_E}{\eta_M} = \frac{C}{C_h} \frac{\eta_{E_{max}}}{\eta_{M_{max}}} \tag{13}$$

According to theory

$$\frac{E}{M} = K \frac{\eta_E}{\eta_M} \tag{14a}$$

and

$$\frac{E_{max}}{M_{max}} = K \frac{\eta_{E_{max}}}{\eta_{M_{max}}} \tag{14b}$$

Here K is a proportionality constant. Combining eq 13, 14a, and 14b gives

$$\frac{E}{M} = \frac{C}{C_h} \frac{E_{max}}{M_{max}}$$

In our case, $E_{max}/M_{max} = 1.14$.

It should be pointed out that this treatment relies on the assumption that the values of E/M produced by vesicles with an asymmetric distribution of pyrene-PC across the bilayer are the same as the values produced from vesicles with a symmetric distribution. Although this is an assumption, it is reasonable because the pyrene moiety of these lipids should be approximately at the midplane of the bilayer.

References

Aune, K. C., Gallagher, J. G., Gotto, M. A., Jr., & Morrisett, J. D. (1977) *Biochemistry 16*, 2151–2156.

Barenholz, Y., Gibbes, D., Litman, B. J., Goll, J., & Thompson, T. E. (1977) *Biochemistry 16*, 2806–2810.

Bartlett, G. R. (1959) *J. Biol. Chem. 234*, 466–468.

Batzri, S., & Korn, E. D. (1973) *Biochim. Biophys. Acta 298*, 1015–1019.

Charlton, S. C., Olson, J. S., Hong, K.-Y., Pownall, H. J., Louie, D. D., & Smith, L. C. (1976) *J. Biol. Chem. 251*, 7952–7955.

Charlton, S. C., Hong, K.-Y., & Smith, L. C. (1978) *Biochemistry 17*, 3304–3310.

Cubero Robles, E., & van den Berg, D. (1969) *Biochim. Biophys. Acta 187*, 520–526.

DeKruijff, B., & van Zoelin, E. J. J. (1978) *Biochim. Biophys. Acta 511*, 105–115.

Doody, M. C., Pownall, H. J., & Smith, L. C. (1978) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 37*, 1832.

Duckwitz-Peterlein, G., & Moraal, H. (1978) *Biophys. Struct. Mech. 4*, 315–326.

Duckwitz-Peterlein, G., Eilenberger, G., & Overath, P. (1977) *Biochim. Biophys. Acta 469*, 311–325.

Förster, Th. (1969) *Angew. Chem. 81*, 361–374.

Galla, H. J., Thielen, U., & Hartmann, W. (1979) *Chem. Phys. Lipids 23*, 239–251.

Kremer, J. M. H., Kops-Werkhoven, M. M., Pathmamanoharan, C., Gijzeman, O. L. J., & Wiersema, P. H. (1977) *Biochim. Biophys. Acta 471*, 177–188.

Lawaczeck, R. (1978) *J. Colloid Interface Sci. 66*, 247–256.

Martin, F. J., & MacDonald R. C. (1976) *Biochemistry 15*, 321–327.

Papahadjopoulos, D., Hui, S., Vail, W. J., & Poste, G. (1976) *Biochim. Biophys. Acta 448*, 245–264.

Roseman, M. A., & Thompson, T. E. (1979) *Biophys. J. 25*, 172-a.

Roseman, M. A., Lentz, B. R., Sears, B., Gibbes, D., & Thompson, T. E. (1978) *Chem. Phys. Lipids 21*, 205–222.

Sengupta, P., Sackmann, E., Kühnle, W., & Scholtz, H. P. (1976) *Biochim. Biophys. Acta 436*, 869–878.

Shaw, J. M., Moore, N. J., Patzer, E. J., Correa-Friere, M., Wagner, R. R., & Thompson, T. E. (1979) *Biochemistry 18*, 538–543.

Thilo, L. (1977) *Biochim. Biophys. Acta 469*, 326-334.
Thompson, T. E. (1978) in *Molecular Specialization and Symmetry in Membrane Function* (Solomon, A. K., & Karnovsky, M., Eds.) pp 78-98, Harvard University Press, Cambridge, MA.
Watts, A., Marsh, D., & Knowles, P. F. (1978) *Biochemistry 17*, 1792-1801.
Wirtz, K. W. A. (1974) *Biochim. Biophys. Acta 344*, 95-117.

Acholeplasma laidlawii Membranes: A Fourier Transform Infrared Study of the Influence of Protein on Lipid Organization and Dynamics†

Hector L. Casal,* David G. Cameron, Ian C. P. Smith, and Henry H. Mantsch

ABSTRACT: Fourier transform infrared spectroscopy is applied to the study of intact and deproteinated plasma membranes of *Acholeplasma laidlawii*, enriched biosynthetically with perdeuteriopalmitoyl chains. The temperature-dependent behavior is monitored via the CD stretching modes and compared with that observed in the model membrane 1,2-diperdeuteriopalmitoyl-*sn*-glycero-3-phosphocholine. The broad ramplike transition observed in the natural membranes is shown to consist of two overlapping stages. In the lower temperature range the principal change is a reduction in the rigidity of the lipid matrix. Subsequently, over a 5 °C range, centered at the growth temperature, a large change in the gauche/trans conformer ratio of the acyl chains occurs, similar to that generally observed in model systems. This is followed by an abrupt cessation of the phase transition. The effects of membrane protein on the phase transition are shown to be relatively minor. Firstly, they produce a decrease in the rate of acyl chain motion at a given temperature, resulting in a reduction in the width of the transition. In addition, the presence of protein increases the population of gauche conformers of the fatty acyl chains in the liquid-crystalline phase of the membrane lipids.

The nature and the state of lipids are believed to regulate not only the architecture and physical properties of biological membranes but also various physiological functions that ensure the viability of the organism; for example, enzyme activities depend to a large extent on the "fluidity" of the lipid matrix (Baldassare et al., 1977; Racker et al., 1975), which, in turn, is a function of temperature and of the lipid composition.

A number of such functional requirements have been ascribed to specific lipid-protein interactions or to the presence of lipoprotein subunits which are believed to form channels for the transport of metabolites or to stabilize the active conformation of lipid-bound enzymes. Such lipid-protein interactions could influence the behavior of the endogenous lipid pool, such as thermotropic mesomorphism, the cooperative change from a solidlike gel state to a fluidlike liquid-crystalline state.

We have focused our attention on *Acholeplasma laidlawii*, a microorganism capable of regulating the "fluidity" of its plasma membrane when the growth temperature or the external fatty acid source is varied (McElhaney, 1974). The enzymatic activity in *A. laidlawii* has been found to depend critically on the phase behavior of the membrane lipid pool, as recently determined with the membrane-bound ATPase (Silvius et al., 1980).

The phase transition of *A. laidlawii* plasma membrane lipids has been studied by different physical techniques such as calorimetry (Steim et al., 1969), X-ray diffraction (Engelman, 1970), NMR[1] (Smith et al., 1979), ESR (Huang et al., 1974; Butler et al., 1978), and IR (Casal et al., 1979). In all cases it was found that the onset of molecular disorder in the lipid fatty acyl chains occurs over a wide temperature range. This is in contrast to model membrane systems where the phase transition is very sharp and complete in less than 1 °C (Sunder et al., 1978). The broad temperature range of the phase transition in *A. laidlawii* membranes could be explained either by lipid-protein interactions which modulate the thermal behavior of the lipid matrix or by heterogeneity of its lipid pool (McElhaney, 1974). Recent studies with *A. laidlawii* membranes enriched to >95% in a particular fatty acid have revealed much narrower phase-transition ranges, supporting the latter explanation (Silvius & McElhaney, 1978).

In order to obtain information regarding the influence of proteins on the degree of organization and the dynamics of the lipid components in a natural membrane, we have performed a detailed Fourier transform infrared study of the plasma membrane of *A. laidlawii* grown on perdeuteriopalmitic acid and of the lipid matrix extracted from the same membrane. This microorganism readily incorporates deuterium-labeled fatty acids into its membrane lipids (Stockton et al., 1975, 1977), providing a direct probe of the membrane structure via the infrared C-D stretching bands. We are able to monitor small variations in these bands and recently demonstrated this ability in a report on the phase transition of *A. laidlawii* grown on perdeuteriopalmitic acid (Casal et al., 1979). In this publication we present a more detailed study of this phase transition and compare it with that observed in the lipids remaining after the removal of the proteins from the membranes and with the behavior of the model membrane DPPC-d_{62}. Considerable insight is obtained into the various types of motion performed by the fatty acyl chains due to the

† From the Division of Chemistry (D.G.C. and H.H.M.) and Division of Biological Sciences (I.C.P.S. and H.L.C.), National Research Council of Canada, Ottawa, Canada K1A 0R6. *Received August 23, 1979.* H.L.C. is supported in part by the University of Ottawa. Issued as NRCC No. 17846.

[1] Abbreviations used: DPPC, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine; DPPC-d_{62}, 1,2-diperdeuteriopalmitoyl-*sn*-glycero-3-phosphocholine; NMR, nuclear magnetic resonance; ESR, electron spin resonance; FT-IR, Fourier transform infrared; DSC, differential scanning calorimetry.

0006-2960/80/0419-0444$01.00/0