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# Lysolipid Exchange with Lipid Vesicle Membranes

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**Abstract**—While the aqueous solubility for bilayer phospholipids is less than  $10^{-10}$  M—keeping lipid membranes at essentially constant mass, single chain surfactants can have a significant aqueous solubility. Thus, in surfactant solutions, both monomer and micelles can interact with a lipid bilayer, and the mass and composition of the bilayer can be changed in seconds. These changes in composition are expected to have direct consequences on bilayer structure and material properties. We have found that the exchange of surfactants like lysolecithin can be described in terms of a kinetic model in which monomer and micelles are transported to the membrane from bulk solution. Molecular transport is considered at the membrane interfaces and across the midplane between the two monolayers of the bilayer. Using micropipet manipulation, single vesicles were transferred into lysolecithin solutions, and the measurement of vesicle area change gave a direct measure of lysolecithin uptake. Transfer back to lysolecithin-free media resulted in desorption. The rates of uptake and desorption could therefore be measured at controlled levels of membrane stress. With increasing lysolecithin concentration in the bulk phase, the amount of lysolecithin in the membrane reached saturation at ~3 mol% for concentrations below the critical micelle concentration (CMC) and at >30 mol% for concentrations above the CMC. When convective transport was used to deliver lysolecithin, uptake occurred via a double exponential: initial uptake into the outer monolayer was fast ( $\sim 0.2 \text{ sec}^{-1}$ ); transfer across the bilayer midplane was much slower ( $0.0019 \text{ sec}^{-1}$ ).

**Keywords**—Lipid vesicles, Lysolecithin, Membrane transport, Membrane permeability, Membrane pores, Micelle forming surfactants, Micropipet manipulation.

## INTRODUCTION

Micropipet methods have been used extensively to characterize lipid bilayer membranes in terms of relations between lipid composition and the thermomechanical properties of the bilayer (19,26,35–38). In particular, experiment and theory have focused on closed vesicle structures in which the mass of material can be considered constant during the course of a mechanical experiment because the aqueous solubility for bilayer phospholipids is so low [less than  $10^{-10}$  M (28)]. Single chain surfactants, on the other hand, have a significant solubility in the aque-

ous phase as monomers and, at higher concentrations can exist as micellar phases. Thus, in solutions of these surfactants both monomer and micelles can be in equilibrium with the lipid bilayer. The mass and composition of the bilayer can now be changed in seconds, which is expected to have direct consequences on bilayer structure and material properties. In this paper, we will discuss the importance of bilayer-soluble molecules, current methods of study, and a general model for surfactant-membrane interactions. These will be followed by a presentation of some recent experiments that use micropipet methods to study: (i) the uptake and desorption of lysolecithin into lipid vesicle membranes; and (ii) the kinetics for various stages of the process. Brief mention will also be made of porous defects that arise due to lysolecithin uptake and other perturbations such as electroporating fields.

## BILAYER-SOLUBLE MOLECULES

A better understanding of the partitioning of exogenous bilayer-soluble molecules into lipid bilayers is of interest because a change of bilayer composition changes both the membrane's barrier properties and its ability to act as a 2D solvent for intramembrane molecules. For example, certain environmental pollutants act on cells by degrading lipids or partitioning into lipid bilayers thereby damaging their membranes (2,27,52); anaesthetics also act via hydrophobic sites in lipids and proteins (15,21); the entry of viruses into cells occurs via special peptides that appear to have an ability to become amphiphilic and partition into the lipid bilayer of the target cell, thus connecting the viral and cellular membranes, and allowing fusion (11); and, in biotechnological applications, cell membrane properties might be selectively modified to optimize certain *in vitro* manipulations (such as electroporation) for gene transfection (51,58).

With regard to natural cellular processes, the exchange or transport of amphiphilic molecules (lipids, fatty acids, cholesterol, bile salts, etc.) into and across bilayer membranes has been a focus of many studies because of their role in metabolism (25). This exchange can be passive (7), in which case, the transported molecule permeates the membrane into the cell interior. Conversely, it may be active, which involves transport proteins (10). As one of

**Acknowledgment**—This work was supported in part by grants GM 40162 and HL23728 from the National Institutes of Health.

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(Received 3 Nov 94, Revised 11 Jan 95, Accepted 11 Jan 95)

the intermediates in these kinds of processes, lysolipids are either degraded or recycled and, through this cycle, are kept in low concentration in cell membranes. In other cellular processes, lipidic surfactants are often second messengers or activators in intracellular and intercellular signaling (5,6). Two examples of particular interest to cell and tissue engineering are in cell-cell adhesion: (i) diacylglycerol is released from membrane phosphoinositolphosphate as part of the cascade that results in the promotion of receptor conformation changes and cytoskeletal assembly; and (ii) the lipidic cytokine, platelet activating factor, is produced by activated endothelial cells and presented such that its influence on integrin receptor avidity is localized to the endothelial cell membrane. Also, cell membranes lining the biliary tract and small intestine are bathed in concentrated solutions of bile acids that should degrade membrane materials (20). As yet, little is known about physical mechanisms of disruption, especially effects on membranes at sub-lytic concentrations of such detergents.

The actions of all these processes involving water-soluble amphiphaths depend on their partitioning into the bilayer membrane phase in relation to other lipid/surfactant-soluble phases that are present. Characterizing the kinetic and equilibrium features of vesicle bilayer-aqueous solution systems is fundamental to understanding the influence of such organic molecules and surfactants on cellular and technological processes.

### CURRENT METHODS OF STUDY

Depending on the chemical nature of the amphiphile, exchange can be considered slow (half-time on the order of hours to weeks), or fast (half-time on the order of seconds to minutes). In general, slow exchange is usually associated with molecules that have a low solubility in the aqueous phase, such as phospholipids, while fast exchange is shown by molecules that readily form micelles. Therefore, several methods, varying in time scale from fractions of a second to days, are required to study the passive exchange of amphiphilic molecules with lipid vesicle membranes. For molecules that are relatively insoluble in aqueous solution, the exchange is studied between donor and acceptor vesicles or liposomes. The most widely used methods that monitor this exchange are radio-labeling and fluorescence labeling (12,22,24,29,32,33,39,41–43,46,48,54–56). In some cases, other methods such as light-scattering (13,14) and free-flow electrophoresis (9) have also proven to be successful for studying molecular exchange with membranes. These studies have found that the rate of uptake and transfer across the bilayer midplane depends both on the chemical characteristics of the transported molecule [such as acyl chain length (14,22,33,48) and headgroup type (22)] and on the composition and packing of the bilayer lipids (12,40).

Of the above methods, only fluorescence and light-scattering are suitable for measuring fast molecular exchange. Of these two, only the fluorescence method can provide additional information about membrane stability by using membrane insoluble probes entrapped inside the vesicle. None of the above methods can control or provide direct information about mechanical stresses in the plane of the membrane. These stresses are important factors for determining the free energy of the bilayer and are thus expected to influence the exchange processes and the formation of defects. The application of controlled stress is, of course, also a requisite for making material property measurements on bilayers that characterize changes in composition-structure-property relations that result from surfactant uptake.

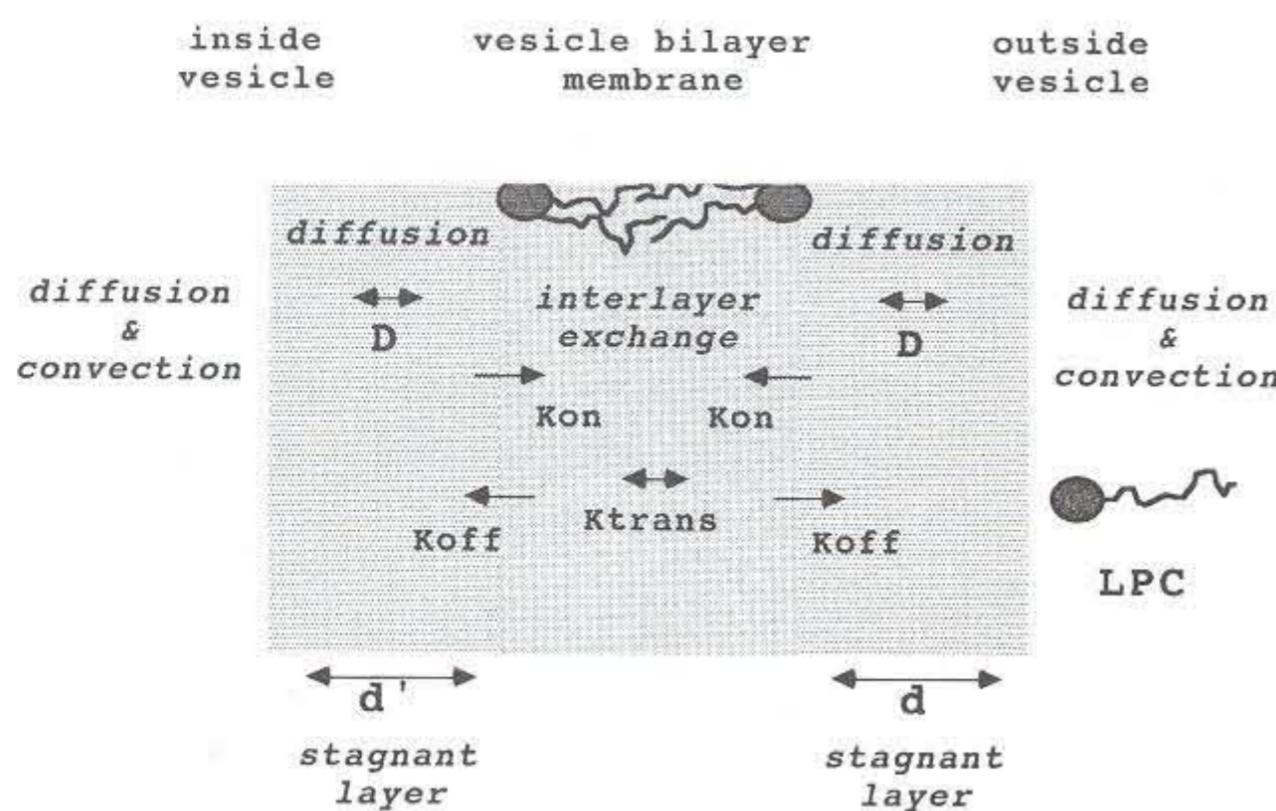
Building on these earlier studies, what is required now are (i) direct measurements of the uptake and desorption of surfactants at controlled levels of membrane stress; and (ii) tests that evaluate the consequences of uptake on bilayer structure and material properties, including the formation and evolution of defects. Here, micropipet methods are currently being developed and used to evaluate such mechanochemical features of the lipid bilayer/surfactant interactions. Following studies that examined the adsorption of tannic acid and its effect on bilayer stability (49), we are now carrying out a series of experiments concerning the effects of lysolecithin on the kinetics of molecular exchange, maximal uptake and porous defect formation for phosphatidylcholine bilayer vesicles (some of which will be reported here). Evans and co-workers are investigating the equilibrium thermodynamics and mechanics of bilayer-surfactant phase equilibria involving the relatively more aqueous-soluble bile acids (20). In related work, the influence of amphipathic viral peptides on vesicle bilayer stability and fusion is being studied for the first time using micropipet manipulation (50).

### KINETIC MODEL

#### *Definitions and Boundary Conditions*

Figure 1 shows a generally accepted scheme that represents the kinetic processes that are expected to be involved in the molecular exchange of a lysolipid<sup>1</sup> with a lipid bilayer vesicle membrane. This exchange essentially involves molecular transport within four different volumes and across the “interfaces” separating these volumes. The four volumes are, the two membranes monolayers, and the two adjacent bathing solutions. The “interfaces” are the apparent outer surfaces of the hydrocarbon regions of the two membrane monolayers that separate the membrane from the aqueous bathing solution, and the mid-plane sur-

<sup>1</sup> Lysolipid, lysophosphatidylcholine, lysoPC, and LPC will all be used interchangeably.



**FIGURE 1.** Schematic diagram of a generalized kinetic model for exchange of lysolipids with bilayer membranes. Lysolecithin (LPC) can be either monomer or micelle depending on concentration.

face of the membrane, which, because of an associated barrier energy for transport, can be considered to separate the two membrane monolayers. Mass transport in the bathing solution is either diffusion limited or is subject to convection. The molecular flux across each "interface" is proportional to the difference in lysolipid concentration on both sides of the "interface" and is characterized by the rates of transport from each of the two bounding volumes. The bilayer and the bathing solution form two regions in series, and each one has its own apparent rate of lysolipid transport.

#### LPC Exchange

With reference to Fig. 1, consider now the molecular exchange for a single lipid vesicle that is brought into a lysolecithin solution. Lysolecithin is initially present in the outside solution where its motion is subject to diffusion and convection. As lysolecithin approaches the bilayer it encounters a stagnant layer of thickness,  $d$ , in which transport occurs by diffusion (characterized by the diffusion coefficient,  $D$ ). Adsorption at the bilayer interface results in intercalation of lysolecithin into the outer monolayer (the overall process is characterized by the "on" rate  $K_{on}$ ). Adsorption-intercalation, or simply uptake,<sup>2</sup> is coupled with desorption of lysolipid from the membrane (given by the "off" rate  $K_{off}$ ). Transfer across the bilayer midplane (characterized by  $K_{trans}$ ) then gives intercalation into the inner monolayer. Finally, desorption from the inner monolayer ( $K_{off}$ ) coupled with adsorption-intercalation ( $K_{on}$ ), and diffusion through the inner stag-

nant layer ( $D$ ) allows the lysolecithin to enter the solution inside the vesicle. The thickness of this internal stagnant layer,  $d'$ , may be different from the one of the outer layer.<sup>3</sup> When the external solution is changed for one that is free of lysolecithin, desorption from the membrane will start from the outer monolayer and the process will be reversed until the bilayer and the vesicle interior are depleted of lysolecithin. The vesicle area changes when the number of intercalated molecules in either of the monolayers changes. Therefore, the overall kinetics can be represented in terms of the amount of lysolipid in the membrane (found directly from micropipet measurements of the instantaneous relative area change of the vesicle) when the area of the transported molecule in the membrane is known.

Based on this general scheme and guided by experimental results from micropipet studies, we have developed a model (Zhelev, submitted for publication), which for a given set of rate constants, quantitatively relates the amount of lysolipid taken up by the membrane at a given time. Both uptake and desorption kinetics depend on the initial concentration of lysolipid in the membrane and in the bulk phases. These kinetics are expected to be affected by factors that reflect both the interaction of the transported molecule with the membrane and its mass transport in the bulk phases. Some of the processes occur in parallel (such as uptake and desorption), while others follow in sequence (such as the transport through the stagnant layer, across the membrane-solution "interface" and across the bilayer midplane). The observed overall kinetics are governed by the fastest rate for parallel processes and by the slowest rate for sequential processes. Therefore, depending on the conditions, the observed "on" and "off" rates may represent actual molecule-membrane interactions or may be dominated by mass transport in the adjacent aqueous phases.

Once at the membrane surface, the initial lysolipid exchange occurs between the outside monolayer and the bathing solution. This apparent rate depends mainly on the rate of desorption (or "off" rate). The "off" rate, in turn, depends on the activation energy of dissociation from the outer monolayer (1,39). From the model it is expected that, when the stagnant layer is not the rate limiting step in the exchange, the rate constants (such as the "on" and "off" rates and the rate of transfer across the bilayer) can be determined from the experimentally measured overall kinetics of uptake and desorption. The theoretically predicted overall kinetics is a sum of two exponentials:

$$N_m = B_0 + B_1 \exp(s_1 t) + B_2 \exp(s_2 t) \quad (1)$$

<sup>2</sup> Uptake includes both adsorption to the aqueous bilayer interface, (involving an attractive interaction between monomer or micelle with the lipid headgroup region of the bilayer), and intercalation of the surfactant molecule in between the lipid molecules of each of the lipid monolayers. Desorption is simply the re-entry of the molecule into the aqueous phase as monomer.

<sup>3</sup> For the size of vesicles employed, and the lysolecithin concentrations we are using ( $\sim 10^{-6}$  M), this effect is negligible because the maximum number of molecules ( $\sim 10^6$ ) in the inside volume ( $3 \times 10^{-12}$  l) is orders of magnitudes smaller than their number in the membrane.

In Eq. 1,  $N_m$  is the instantaneous number of intercalated molecules in the membrane; the coefficients,  $s_1$  and  $s_2$ , are constants related to the "off" rate and the rate of interbilayer transfer; and  $t$  is the time. The coefficients,  $B_0$ ,  $B_1$ , and  $B_2$ , depend on the rate constants and on the initial concentration of lysolipid in the membrane and in the bathing solution. Thus, these coefficients will vary depending on experimental conditions. In the simplest case of slow rates of exchange (no stagnant layer) and when the rate of interbilayer transfer is much smaller than the "off" rate, the coefficients of Eq. 1 are:

$$s_1 = -K_{\text{off}} \text{ and } s_2 = -K_{\text{trans}}; B_0 = \frac{2A_m K_{\text{on}} C_\infty}{K_{\text{off}}},$$

$$B_1 = N_{imo} - \frac{B_0}{2}, \text{ and } B_2 = N_{imi} - \frac{B_0}{2},$$

where  $A_m$  is the area of the vesicle membrane exposed to the solution containing lysoPC,  $C_\infty$  is the concentration of lysoPC in the bathing solution and  $N_{imo}$  and  $N_{imi}$  are the initial numbers of lysoPC molecules in the outside and inside monolayers, respectively.

When the exchange is affected by diffusion across the stagnant layer,<sup>4</sup> the number of intercalated molecules is also given by the sum of two exponentials (with different constants), and so the measured overall kinetics in this case do not provide information about the dependence of the apparent rate of exchange on the interfacial interaction. Therefore, additional information, such as activation energy for transfer, is needed to compare the true "off" rate with the apparent rate of exchange.

#### *Predictions of the Model for Lysolecithin*

Some of the pertinent predictions of the kinetic model will now be discussed.

**Concentration Effects.** The difference between the lysolecithin concentration in the bathing solution and in the membrane sets the initial gradient for diffusion. The amount of uptake under stationary conditions is concentration dependent, both for surfactant concentration below the critical micelle concentration (CMC) and above the CMC. Thus, as our experimental results show, the amount of lysolipid in the membrane can be controlled up to its saturation limit, at which point the membrane lipids are essentially dissolved into a micellar phase.

**Diffusive and Convective Transport.** The transport of lysolipid to the membrane surface depends both on its diffusion and its convection in the bathing solution. For

pure diffusion, the bathing solution around the vesicle can be significantly depleted and so the boundary concentration of lysolipid can change during uptake. When mass transport is dominated by convection (which is achieved experimentally by delivering lysolipid with a flow pipet) a constant boundary concentration can be maintained. The parameter,  $\lambda$ , that characterizes the "stagnant" or "stationary" layer is defined in terms of the "on" rate  $K_{\text{on}}$  as:

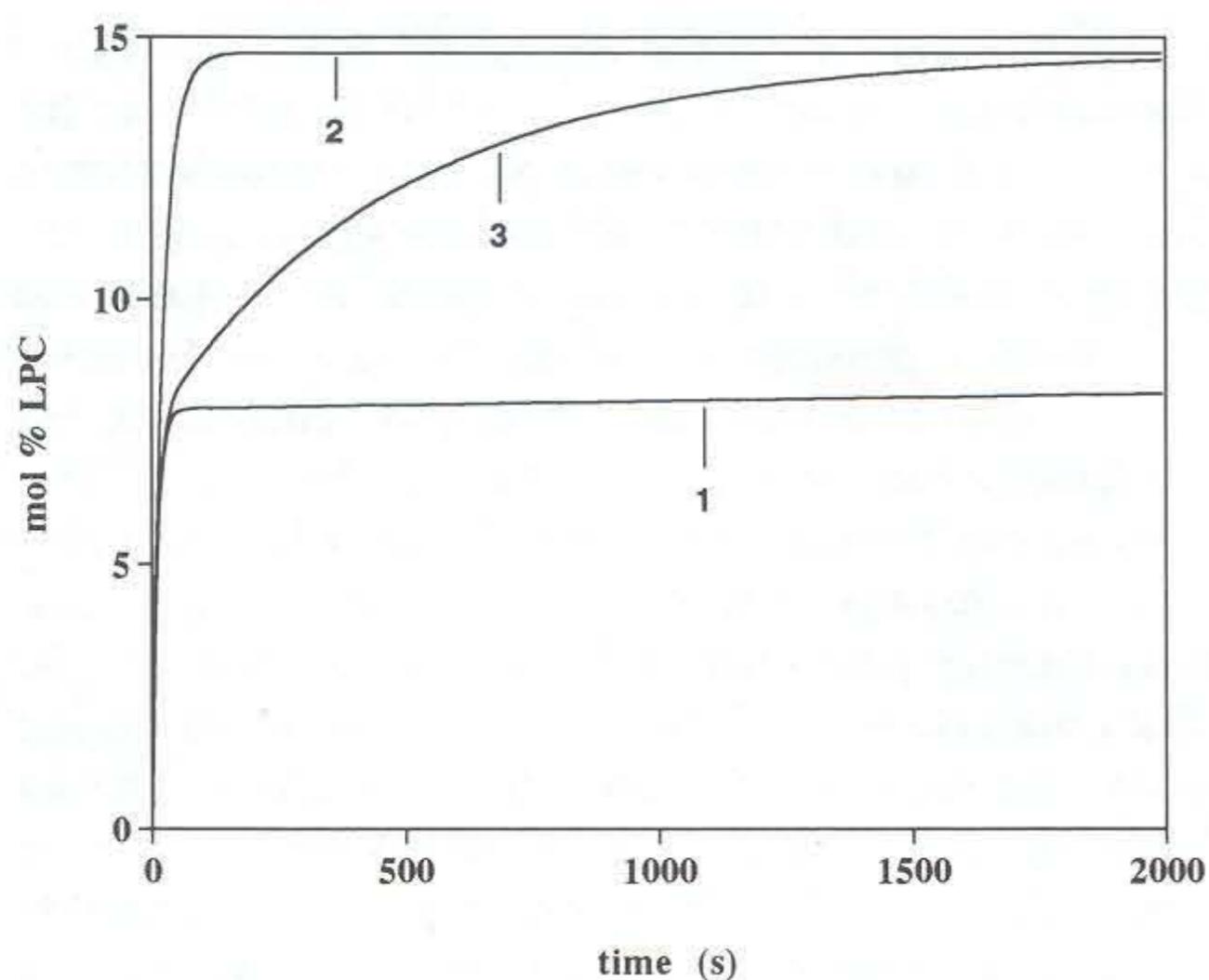
$$\lambda = \frac{K_{\text{on}} A_m}{p} \quad (2)$$

where  $A_m$  is the vesicle area; and  $p$  is the mass transfer coefficient for the stagnant layer which depends on the vesicle size, the diffusion constant, and the flow velocity. The effect of the stagnant layer is negligible when  $\lambda < 1$ . From Eq. 2, it follows that for a given vesicle size and solution concentrations, the apparent thickness of the stagnant layer can be reduced by increasing the fluid velocity tangent to the membrane surface, and the apparent rate of transport from the solution to the membrane is expected to increase significantly.

Clearly, in order to determine the exchange rates at the bilayer surface and the rate of transfer across the bilayer midplane, this layer thickness must be sufficiently small that the apparent rate of transport across the stagnant layer is faster than the rates of exchange. As we will describe later, it appears that, with levels of flow of lysolecithin solution permissible in experiment, the true "on" and "off" rates are inaccessible to measurement because of the dominance of the stagnant layer.

**Outer Monolayer Exchange and Transfer to Inner Monolayer.** The amount and rate of uptake into the outer monolayer and the transfer of lysolipid to the inner monolayer is best discussed in terms of certain limiting cases. As shown in Fig. 2, the kinetics are a single exponential when the rate of transfer across the bilayer mid-plane is either much larger or much smaller than the "on" and "off" rates. When much smaller (Curve 1 of Fig. 2), the inner monolayer is not "seen" and uptake is only into the outer monolayer; when much larger (Curve 2 of Fig. 2), the two monolayers always have the same lysolipid concentration and essentially represent a single sink. For the intermediate case (Curve 3 in Fig. 2), uptake follows a double exponential with a fast uptake into the outer monolayer and a slower transfer across the bilayer midplane. Under these conditions, the "off" and "on" rates can, in principle (when not dominated by the stagnant layer), be found from the experimentally measured fast rate of the double exponential and the corresponding "apparent" partition coefficient. The slow rate of the double exponential is an estimate for the rate of transfer across the bilayer midplane. Assuming that the inner monolayer can expand while uptake proceeds to fill-up the outer monolayer, the

<sup>4</sup> A third exponential term,  $B_3 \exp(s_3 t)$  gives the exchange with the vesicle interior, and as discussed earlier can be neglected for the vesicle case.



**FIGURE 2.** Molar concentration of lysolipid in the membrane calculated from the model for uptake. (1) The rate of transfer across the bilayer midplane is much smaller than the "on" and "off" rates and only the outer monolayer fills. (2) The rate of transfer across the bilayer midplane is much larger than the "on" and "off" rates and both monolayers fill. (3) Uptake follows a double exponential with a fast uptake into the outer monolayer and a slower transfer across the bilayer midplane.

apparent saturation limit for the first exponential might be expected to be half of the true saturation limit because the outside monolayer bares half of the total amount of lysolipid partitioning in the membrane at equilibrium. However, as the experimental results will show, expansion of the inner monolayer and the apparent formation of LPC "transport defects" seems to cause redistribution of LPC before half saturation is reached. These purported defects may arise because the intercalation of molecules in the outside monolayer leads to an increase of its area, which causes a commensurate increase in the area of the unmodified inside monolayer. This increase in area of the inside monolayer, while its number of molecules remains almost constant, creates an apparent monolayer tension (and induced bending moment). From mechanical experiments on vesicle bilayers (38,49), it might be expected that, when the increase in the fractional area per molecule in the inside monolayer reaches ~5% or so, the membrane will break. If the membrane area increases beyond this critical areal strain, some kind of "transverse defect" must form that facilitates the lysolecithin transport across the midplane and relaxes this tension, thereby equilibrating the two membrane monolayers. (The presence of membrane defects is also expected to reduce significantly membrane mechanical stability.)

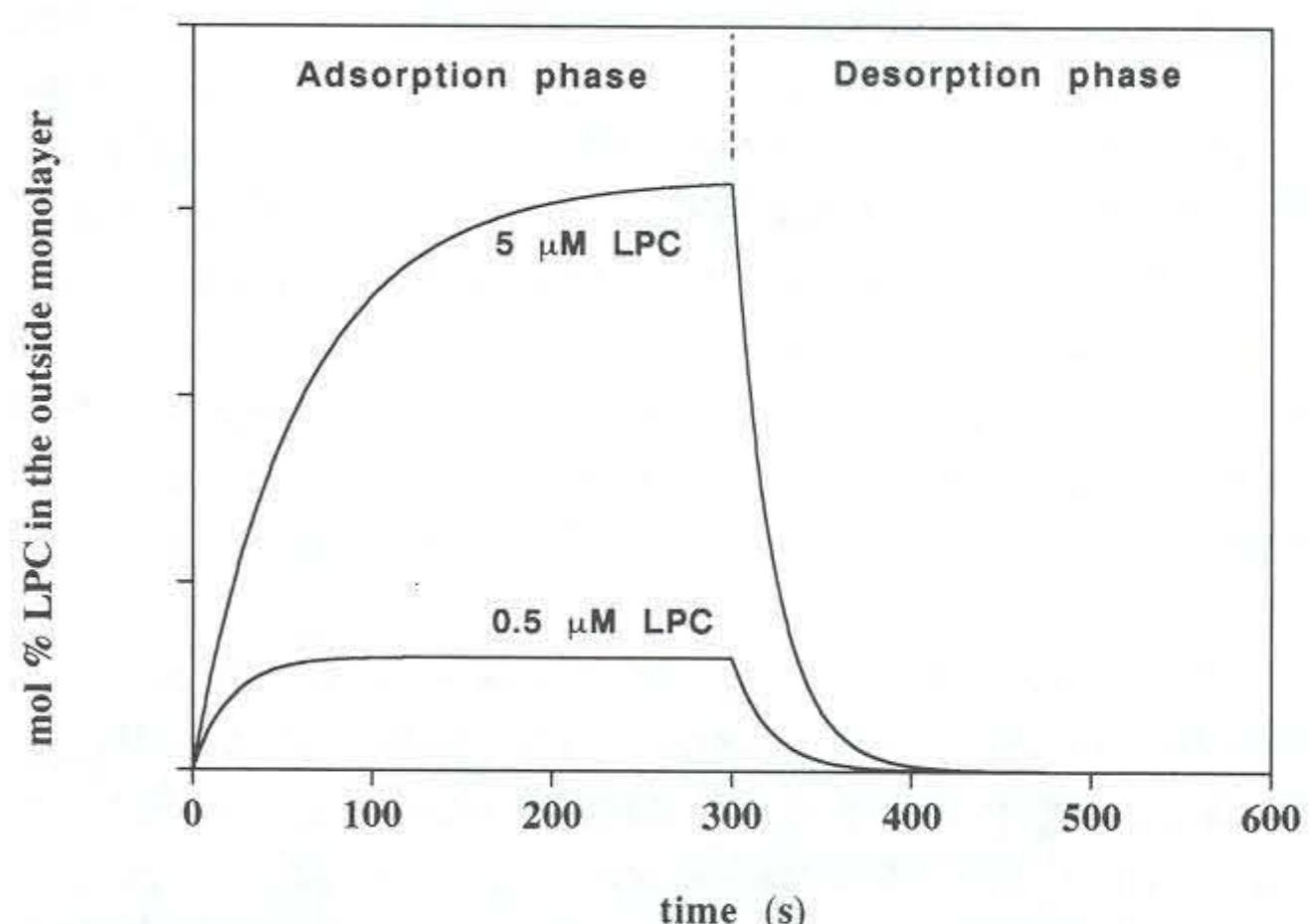
Desorption kinetics for the saturated bilayer is also expected to be a double exponential with correspondingly faster and slower components than for uptake kinetics.

*Monomer vs Micelle.* Because lysolipid can be both mo-

nomic and multimeric (as micelles) the presence of the stagnant layer is expected to significantly influence the observed overall kinetics for concentrations of lysolipid below and above the CMC. Below the CMC, the apparent rates of uptake and desorption are expected to be similar for similar flow conditions. Above the CMC, the uptake of lysolipid may follow two pathways: one by monomer intercalation and other by micelle break-up/fusion at the membrane surface. The rates of uptake and desorption could therefore be different because the larger micelles are transported to the vesicle surface with a smaller diffusion coefficient than monomers and upon reaching the interface must disassociate.

The different expected behavior for uptake of lysolipid into the outer monolayer of the membrane is shown in Fig. 3. As introduced earlier (Eq. 2), transport of each species across the stagnant layer is represented by the parameter,  $\lambda$ . At concentrations below the CMC ( $0.5 \mu\text{M}$ ), the apparent "on" rate for monomers is on the order of  $10^{-4} \text{ cm} \cdot \text{sec}^{-1}$ : the vesicles used in micropipet experiments have radii of  $10 \mu\text{m}$ ; and the diffusion constant of lysolipid monomers is on the order of  $5 \times 10^{-6} \text{ cm}^2 \cdot \text{sec}^{-1}$ . Thus, for a flow velocity of  $50 \mu\text{m/sec}$ , the parameter  $\lambda$  is on the order of 0.3, and the overall kinetics of uptake and desorption are expected to be equal.

At concentrations above the CMC ( $5 \mu\text{M}$ ), adsorbing species include both monomers and micelles, while desorption into lysolecithin-free media involves only monomers. The micelle's apparent "on" rate is on the order of  $10^{-3} \text{ cm} \cdot \text{sec}^{-1}$  and the value for  $\lambda$  for the same  $50 \mu\text{m/sec}$  flow velocity is 12. It is therefore expected that,



**FIGURE 3.** Instantaneous molar concentration of lysolipid in the outside monolayer for different bulk concentrations of lysolipid below ( $0.5 \mu\text{M}$ ) and above ( $5 \mu\text{M}$ ) the critical micelle concentration as predicted by the kinetic model. The parameters used in the model are: "on" rate =  $3 \times 10^{-3} \text{ cm} \cdot \text{sec}^{-1}$  for monomers and  $1.5 \times 10^{-3} \text{ cm} \cdot \text{sec}^{-1}$  for micelles; mass transfer coefficient for the stagnant layer =  $3 \times 10^{-8} \text{ sec} \cdot \text{cm}^{-3}$  for monomers and  $4 \times 10^{-9} \text{ sec} \cdot \text{cm}^{-3}$  for micelles; "off" rate =  $0.2 \text{ sec}^{-1}$ .

when the stagnant layer dominates the overall kinetics (especially for slow flow rates), uptake will have a smaller apparent rate than desorption as depicted in Fig. 3. As discussed earlier and shown here, the model also predicts that the amount of lysolipid in the outer monolayer will be higher for the higher concentration.

### MICROPIPET EXPERIMENTATION

The task is to devise experiments that provide kinetic and equilibrium parameters that characterize each phase of the exchange. In the next sections, some recent experiments will be presented that have begun to test the various phases of uptake and desorption. In the experiments described, two parameters are varied: the concentration of lysolecithin in the bathing solution, and the rate at which the lysolecithin solution is delivered to the vesicle membrane. Lysolecithin exchange is detected by area changes of the lipid vesicle membrane.

#### *Materials and Methods*

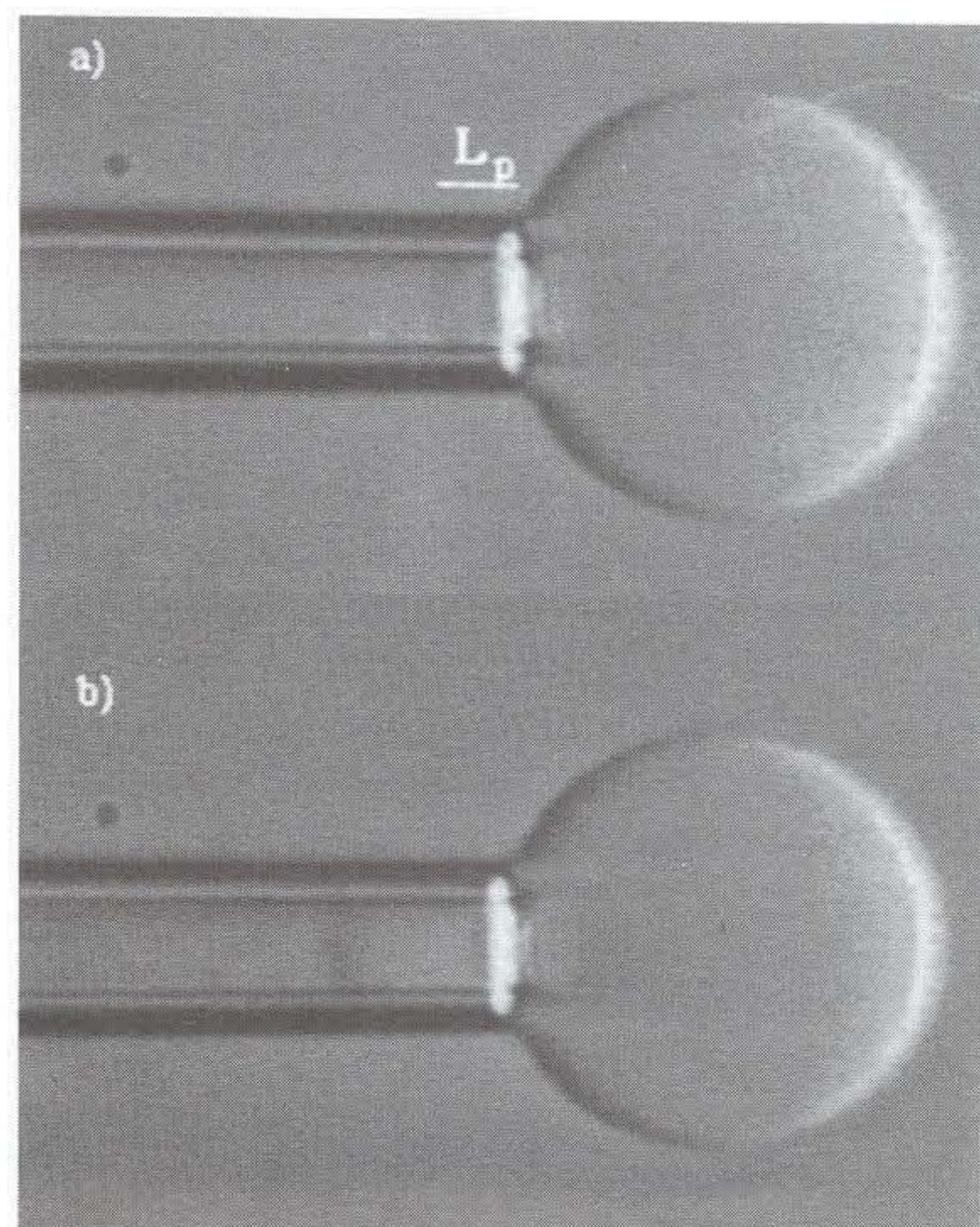
The methods used in the lysolecithin exchange experiments were based on the micropipet manipulation techniques that we have developed for measuring the material properties of lipid vesicle bilayer membranes. The preparation of lipid vesicles from stock organic lipid solutions and the preparative procedures for a range of lipid and mixed lipid systems are documented in recent publications (35,36). Egg phosphatidylcholine (EPC) (Avanti Polar Lipids) was used as the lipid for all experiments and the lysolipid was monooleylphosphatidylcholine (MPC). Giant (20- to 40- $\mu\text{m}$  diameter), single walled vesicles were formed by rehydrating dried lipid with a 160 mOsm sucrose solution. Once formed a sample of vesicles was resuspended in equiosmotic (160 mOsm) glucose solution in the microscope chamber. The density and refractive index difference between these two solutions ensures that the vesicles settle to the bottom of the chamber and are more readily visualized by interference microscopy for ease of capture. Using the micropipet, single walled vesicles are selected from the largely multilamellar population.

The experimental set-up and equipment have been documented in recent publications (16–18,34,35). Briefly, a glass micropipet of desired internal diameter and flat tip was formed by pipet puller and cut by a quick fracture using a microforge. The pipet was used to both apply the force to the aspirated vesicle during the vesicle deformation tests and to measure the resulting deformation. The measuring micropipet was mounted in a micromanipulator (Research Instruments Inc., Durham, NC), via a “wet” chuck that serves to connect the pipet to the water-filled manometer system that controls the pipet pressure (34,35).

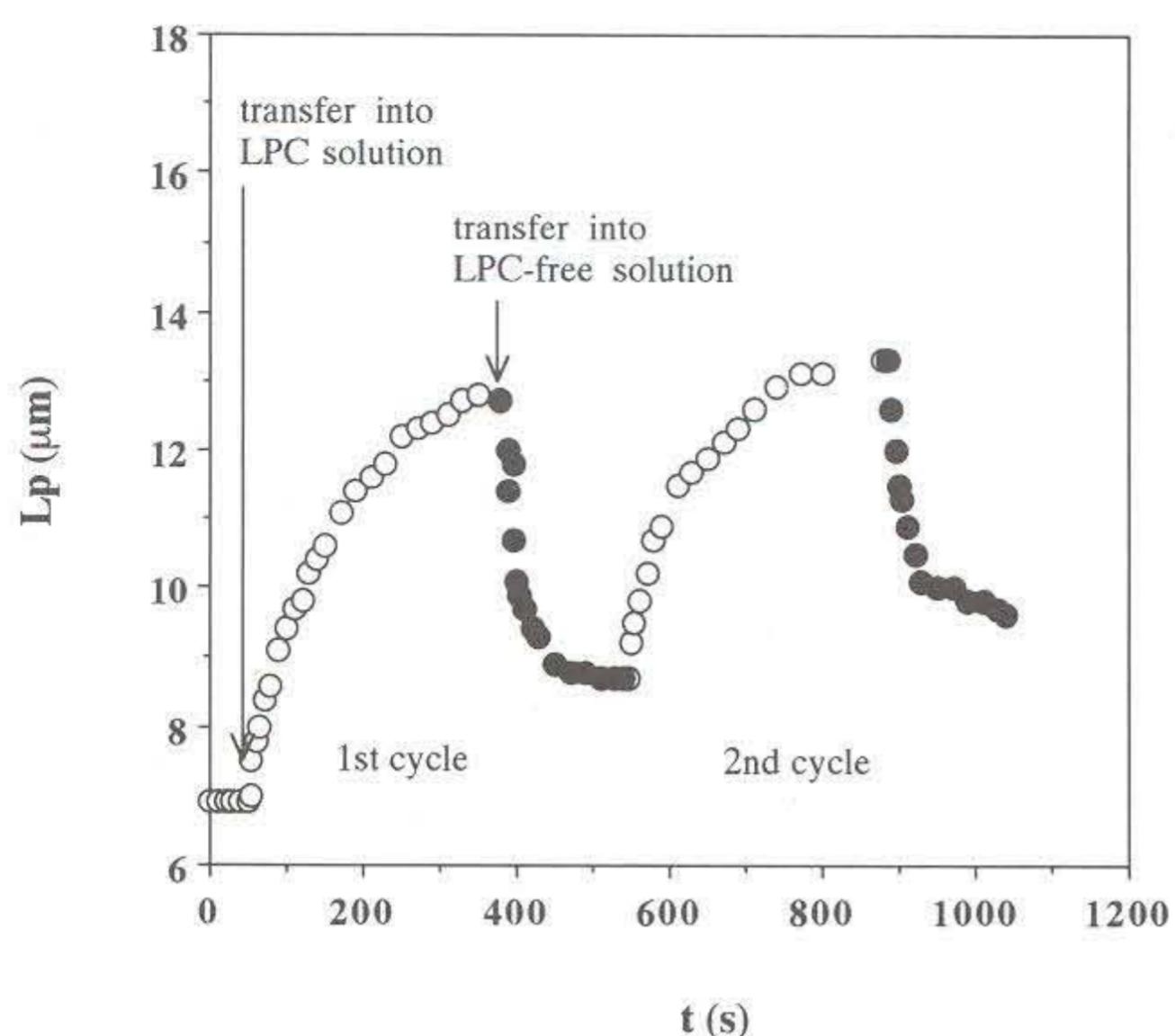
The manipulator allowed the pipet to be held absolutely stationary (resolution < 1  $\mu\text{m}$ ) and to be moved in the axial, lateral, and vertical directions by the transduction of three separate and variable air pressures provided by the joy stick via flexible transmission tubes. Both coarse and fine control is possible. The whole assembly was mounted on the microscope stage so that the pipet entered the microchamber horizontally (34). In these experiments, vesicles were either transferred into an adjacent microchamber on the microscope stage by using a large (100- $\mu\text{m}$  diameter) transfer pipet that spans the air gap between the chambers and protects the measuring pipet and vesicle during the transfer; or a flow pipet was used to rapidly exchange and maintain a flow of isoosmotic solution around the test vesicle. This flow pipet was connected to the standard water-filled manometer system, so the flow of solution from the pipet was accurately controlled by displacement of the water reservoir. For example, a pressure difference of 20 N/m<sup>2</sup> produced a flow rate of 240  $\mu\text{m/sec}$  at the tip of the 40- $\mu\text{m}$  flow pipet.

#### *Lysolipid Exchange with a Single Vesicle Membrane*

*The Basic Exchange Experiment.* In the basic exchange experiment shown in Fig. 4, a single vesicle was supported with constant suction pressure while it was trans-



**FIGURE 4.** Videomicrographs of a 20  $\mu\text{m}$  diameter vesicle held under constant, low, membrane tension (0.5 mN m<sup>-1</sup>) (a) before and (b) after transfer into a solution containing lysolecithin. The increase of the vesicle projection length inside the pipet corresponds to 2% increase of its membrane area.



**FIGURE 5.** Uptake and desorption cycles for lysolecithin exchange measured by the change of the projection length ( $L_p$ ) of the vesicle membrane inside the pipet upon transferring the vesicle between two chambers: one containing the lysolipid at 1  $\mu\text{M}$ , and the other containing an equiosmotic solution that is free of lysolecithin.

ferred between two microchambers containing iso-osmotic solutions that were lysolecithin-free or had lysolecithin at some desired concentration. We observed an increase in the projection length ( $L_p$ ) of the vesicle membrane inside the pipet upon transfer into the lysolecithin solution. The time course of this change is shown in Fig. 5. Upon transferring the vesicle into the solution containing lysolipid (in this case, 1  $\mu\text{M}$  lysolipid) the  $L_p$  of the vesicle membrane inside the pipet increased and eventually reached a plateau over a period of 300 to 400 sec. When the vesicle was transferred back into the lysolipid free solution, the projection length decreased immediately and almost reached its initial value in 100 to 200 sec. This cycle of uptake (adsorption and intercalation) and desorption could be repeated several times, which demonstrates the reversibility of lysolecithin uptake. The observed changes of the vesicle projection length were a result of the change of vesicle area at constant vesicle volume. For this particular case, the change in projection length at saturation was  $\sim 6 \mu\text{m}$ . If the vesicle maintained constant volume, this length change was proportional to the relative change in vesicle membrane area  $\Delta A$  (26) and corresponded to a relative area increase of  $\sim 2\%$ . This area increase was converted into a molar concentration of lysolipid in the vesicle membrane when the area per molecule of the bilayer lipid and the lysolipid are known. Since the area per EPC molecule in the bilayer membrane is  $64 \text{ \AA}^2$  (31) and that for MPC is  $44 \text{ \AA}^2$  (23,30), the area increase of 2% corresponded to a membrane uptake of 3.3 mol% MPC. An important feature of the experimental technique is that during this whole

process the membrane tension was controlled via the pipet suction pressure. In the present example, the suction pressure in the pipet was kept constant at  $800 \text{ N/m}^2$ , which converts to a constant membrane tension of 1.5 mN/m.

The accumulation of lysolecithin under these static conditions was dominated by diffusion in the bathing solution. When lysolecithin desorbed, the driving force was the concentration gradient from bilayer to solution and the area decreased. The final vesicle area returned to a value that is close to the area before uptake. For concentrations at and below 1 micromolar, only a few mol% LPC were taken up by the membrane, and, for the time scale of this experiment, the uptake followed a single exponential. From the model, this single exponential reflected uptake only by the outer monolayer of the bilayer (i.e., there was negligible transfer of lysolecithin or phospholipid across the midplane). As will be discussed later, at higher lysolipid concentrations this was not always the case and redistribution into the inner monolayer occurred. Thus, this simple transfer experiment provides a direct measure of the extent and rate of uptake of lysolecithin upon exposure to a lysolecithin solution and similarly measures the rate and extent of desorption for the same vesicle. The method gives the whole time history of lysolecithin exchange for a single bilayer vesicle with controlled membrane tension.

The above experiment was carried out at a concentration of 1  $\mu\text{M}$  lysolecithin. The critical micelle concentration of this particular monooleoyl lysolecithin was measured by standard surface monolayer methods and found to be approximately 1  $\mu\text{M}$  in agreement with published data (28). As discussed earlier, it might be expected that the rate and extent of exchange would be different at concentrations above the CMC, such as 5 or 10  $\mu\text{M}$ . Figure 6 shows a comparison between the three concentrations, again for transfer into and out of lysolecithin solutions. If monomers are the only species that are intercalated into the vesicle membrane the initial rate of increase of the vesicle area as well as the maximum increase of this area during uptake are expected to be the same for all concentrations above the CMC. However Fig. 6 shows that the initial rates of uptake and the molar concentration for "saturation" (found from the extrapolated experimental points) were in fact larger for higher concentrations. This result demonstrated that the transport of lysolipid into the membrane occurred both by uptake of monomers and by fusion of micelles with the bilayer.

The uptake and desorption kinetics in all three cases were fitted with a single exponential, again suggesting a single rate limiting process. When the rate limiting process is exchange of lysolecithin between the solution and the bilayer interface, the model predicted that the rate of uptake should be similar to the rate of desorption. However, for these static cases we found that the rate of uptake was always smaller than the rate of desorption. This result

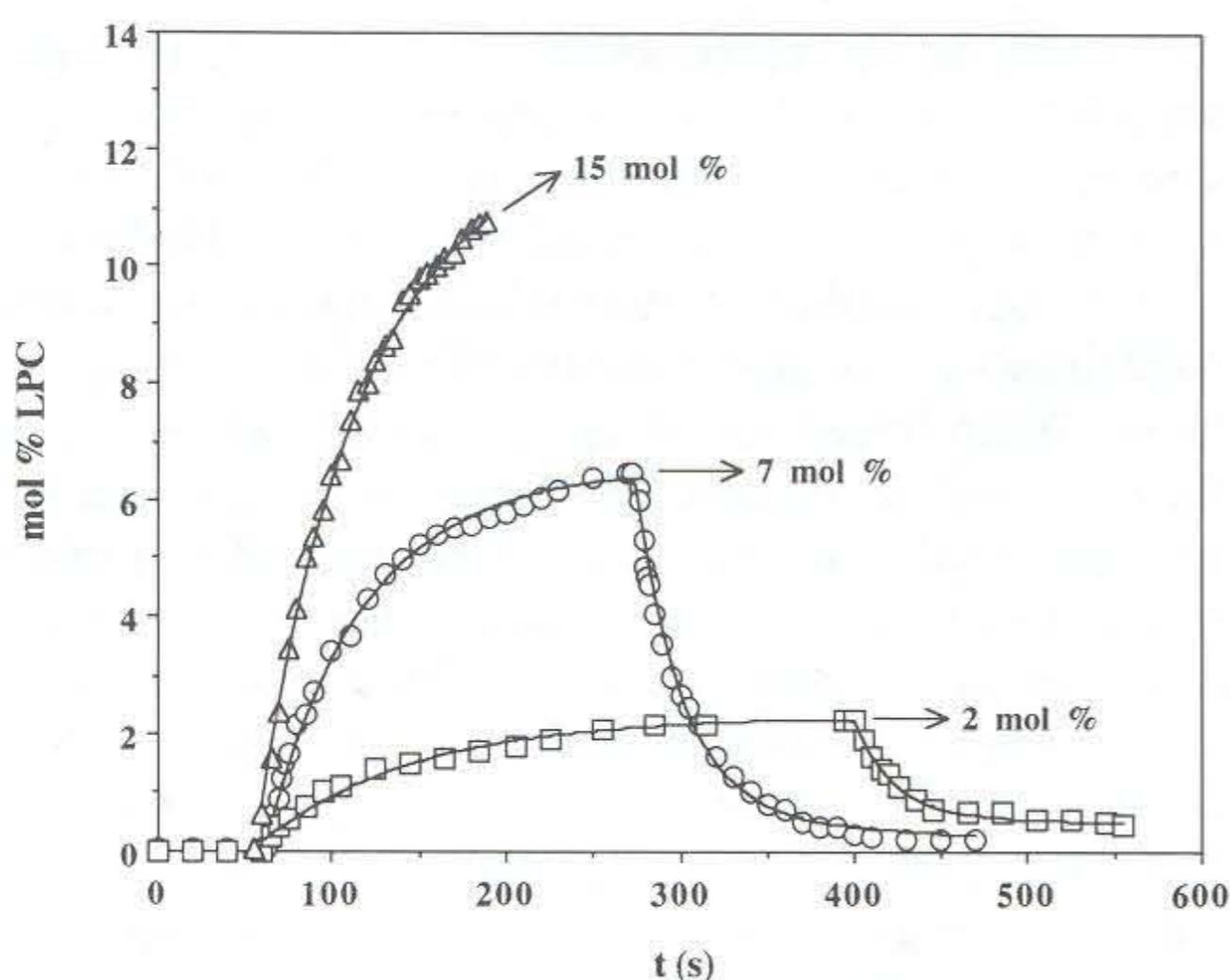


FIGURE 6. Lysolecithin exchange in solutions containing different concentrations of lysolecithin. (squares) 1  $\mu\text{M}$ ; (circles) 5  $\mu\text{M}$ ; and (triangles) 10  $\mu\text{M}$  concentration of the lysolipid, respectively. Also shown are the extrapolated membrane concentrations of lysolipid at equilibrium.

suggests that molecular transport under static conditions is in fact diffusion limited and we were not measuring the actual rate for exchange with the outside monolayer but the slower transport of LPC to the surface. During uptake, it seems that the concentration of lysoPC around the vesicle was depleted and uptake slowed, while desorption was more rapid upon transferring the vesicle to a lysolecithin-free solution. A constant boundary condition for lysolecithin concentration at the bilayer surface is therefore required; it is also desirable to reduce as much as possible the stationary layer. The next experiment addresses these points.

*Exchange with Controlled Flow Rate.* In order to establish a constant boundary condition for lysolecithin concentra-

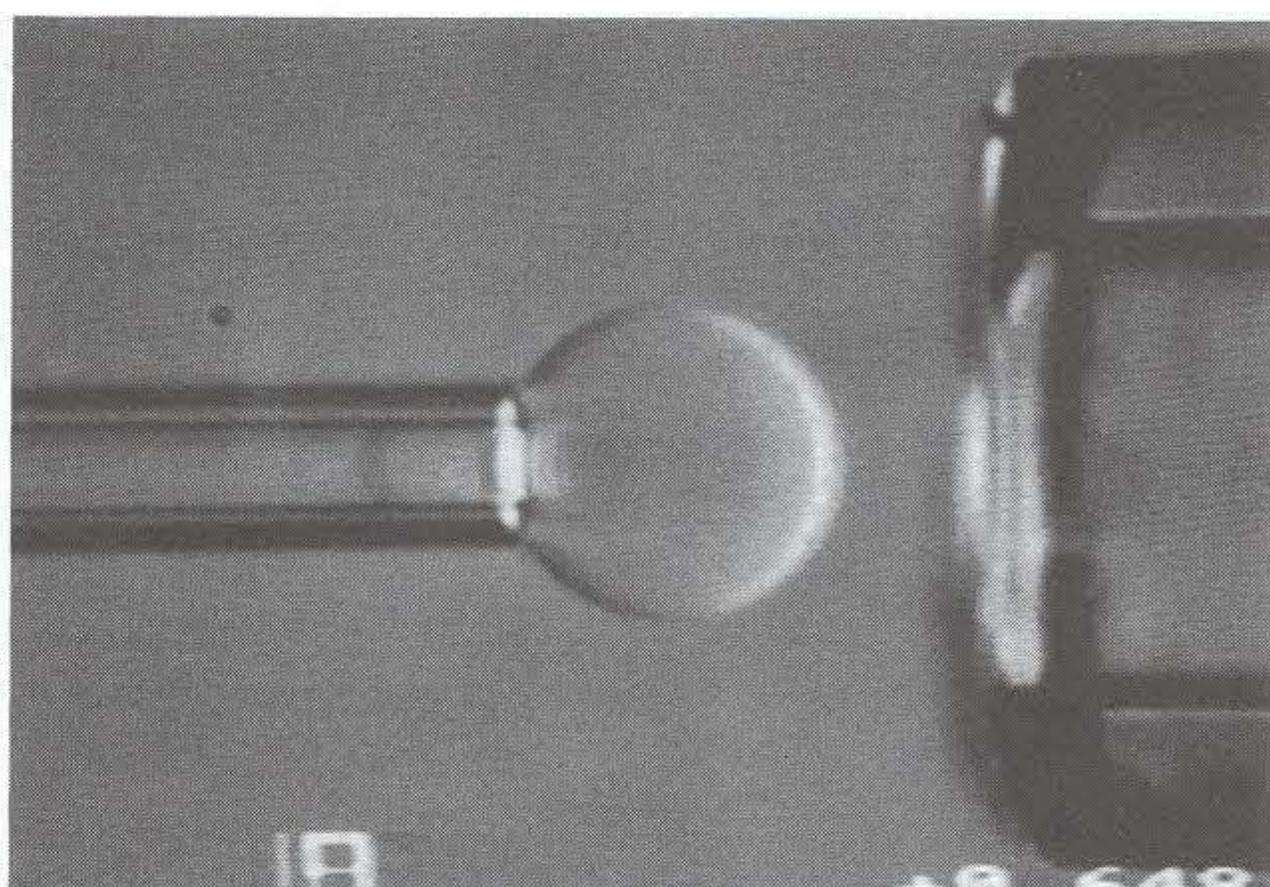


FIGURE 7. Videomicrograph showing arrangement of the 40- $\mu\text{m}$  diameter flow pipet on the right, which delivers LPC solution at a controlled flow rate to the test vesicle.

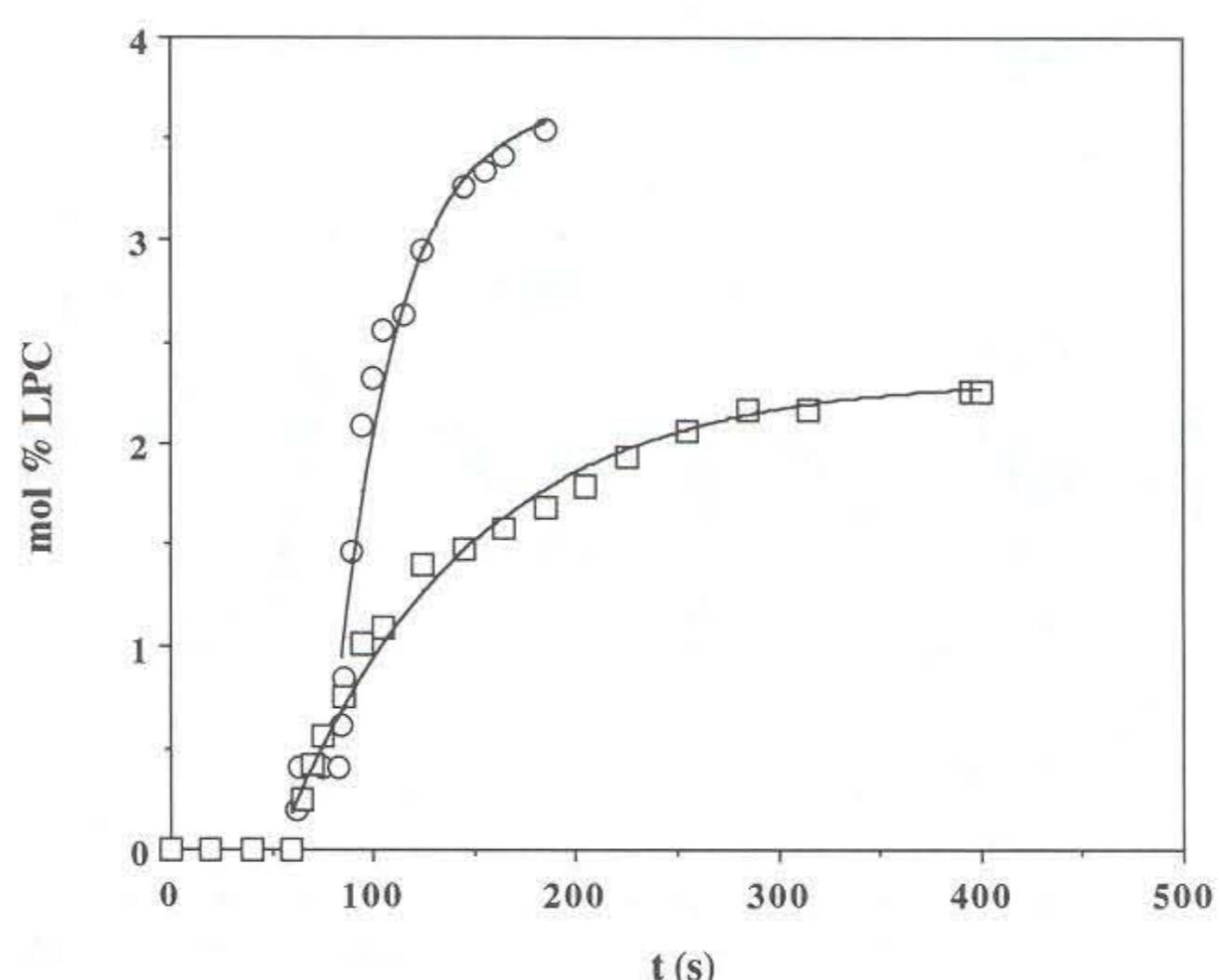


FIGURE 8. Uptake of lysolecithin by a lipid vesicle from a solution of 1- $\mu\text{M}$  lysolecithin comparing stationary and flow conditions. (squares) Zero flow velocity (rate of uptake =  $-0.011 \text{ sec}^{-1}$  and maximal uptake = 2.4%); (circles) flow velocity of  $250 \mu\text{m} \cdot \text{sec}^{-1}$  (rate of uptake =  $-0.032 \text{ sec}^{-1}$ ; and maximal uptake = 3.7%).

tion at the bilayer surface and to reduce the influence of the stationary layer, a micropipet flow method was used to deliver solution directly to a vesicle at known rates of flow. As shown in Fig. 7 the vesicle in the micropipet was held stationary and a second, larger pipet was brought up to the test vesicle already having a preset flow rate of lysolecithin solution.

Data obtained for lysolecithin exchange at 1  $\mu\text{M}$  concentration using this flow pipet are shown in Fig. 8. With flow of solution over the vesicle, a much more rapid uptake is achieved. Both curves were again fitted with a single exponential. The rate of LPC uptake and its apparent saturation limit for stationary conditions were  $-0.011 \text{ sec}^{-1}$  and 2.4%, and for flow conditions were  $-0.032 \text{ sec}^{-1}$  and 3.7%, respectively. Upon desorption of lysolecithin, both vesicles returned to their original length in the pipet (not shown) demonstrating that the changes in projection length were all area changes. This directly demonstrates that the depletion of LPC from the solution around the vesicle under stationary conditions is an important rate determining factor. Thus, at 1  $\mu\text{M}$  lysolecithin, flow produced a rate of uptake approximately three times faster than static transfer, while the two conditions produced approximately the same lysolecithin concentration in the membrane at equilibrium (2.4 mol% and 3.7 mol%).

*LPC Desorption and the True "Off" Rate.* The average desorption rate for vesicles in moderate flow (300  $\mu\text{m/sec}$ ) was approximately  $0.2 \text{ sec}^{-1}$ . As suggested earlier, in order to address the question of whether, even with flow, we were measuring the true "off" rate for lysolipid or the

experimentally measured rate was still affected by diffusion across the stagnant layer, an estimation of the activation energy for desorption was made and compared to other studies and theoretical predictions. The "off" rate is related to the activation energy for dissociation which can be calculated using the method of Aniansson et al. (1). The true "off" rate  $K_{mb}$  for dissociation from the membrane and the activation energy of dissociation  $\Delta G$  are related by,

$$K_{mb} = \frac{D(\Delta G)^2}{(KTl_{max})^2} \exp\left(-\frac{\Delta G}{KT}\right) \quad (3)$$

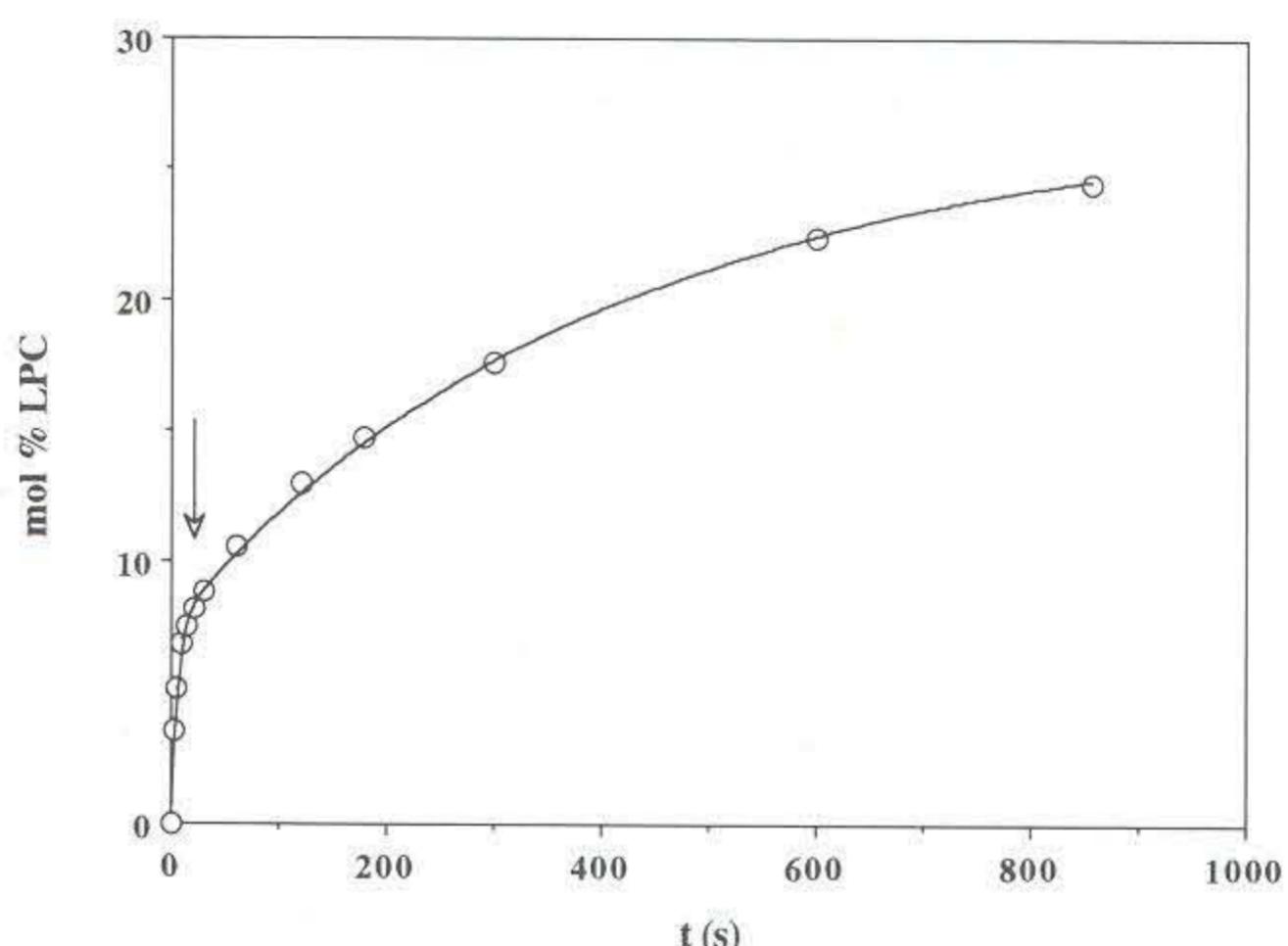
where  $D$  is the diffusion coefficient of the monomer in the bathing solution ( $5 \times 10^{-6} \text{ cm}^2/\text{sec}$ ),  $K$  is the Boltzmann constant,  $T$  is absolute temperature, and  $l_{max}$  is the maximum length of the monomer hydrocarbon chain (17.4 Å). From our average measured desorption rate of  $0.2 \text{ sec}^{-1}$ , an activation energy for dissociation was calculated to be  $\sim 1.12 \times 10^{-19} \text{ J}$ . For the sake of comparison with other values, this converts (from J to kcal) to 0.91 kcal/hydrocarbon group. This value is larger than the value of 0.78 kcal/hydrocarbon group measured for phosphatidylcholine lipids (39), and that for dimyristoylphosphatidylethanolamine of 0.8 kcal/hydrocarbon group measured by Wimley and Thompson (55). Although these activation energies appear to be similar, the difference of  $\sim 0.1$  kcal/hydrocarbon group represents a one to two orders of magnitude slower rate for desorption for our experiments. This suggests that, even in moderate flow, the desorption of lysolipids is still dominated by diffusion across the stagnant layer and the true "off" rate cannot be measured. In the Nichols and Wimley and Thompson experiments the true "off" rate could be measured because in these cases the phospholipid molecules had a much lower solubility in the aqueous phase and the desorption step was apparently much slower than diffusion across the stagnant layer. These results and comparisons illustrate the differences that exist between different bilayer soluble molecules and demonstrate the kind of problems that exist in attempting to make experimental measurements of desorption kinetics in these systems.

**Maximal LPC Uptake and Intrabilayer Transfer.** The phase diagram for monooleoyllysolecithin in dioleoylphosphatidylcholine shows a solubility limit for monooleoyllysolecithin of almost 50 mol%, after which point the bilayer transforms into a micelle phase (53). It is therefore of interest to determine whether the maximal uptake of lysolipid for a single vesicle can attain this value. As predicted by the model, the uptake of lysolipid in the membrane is increased by increasing the concentration of the surfactant in the bathing solution. In order to achieve

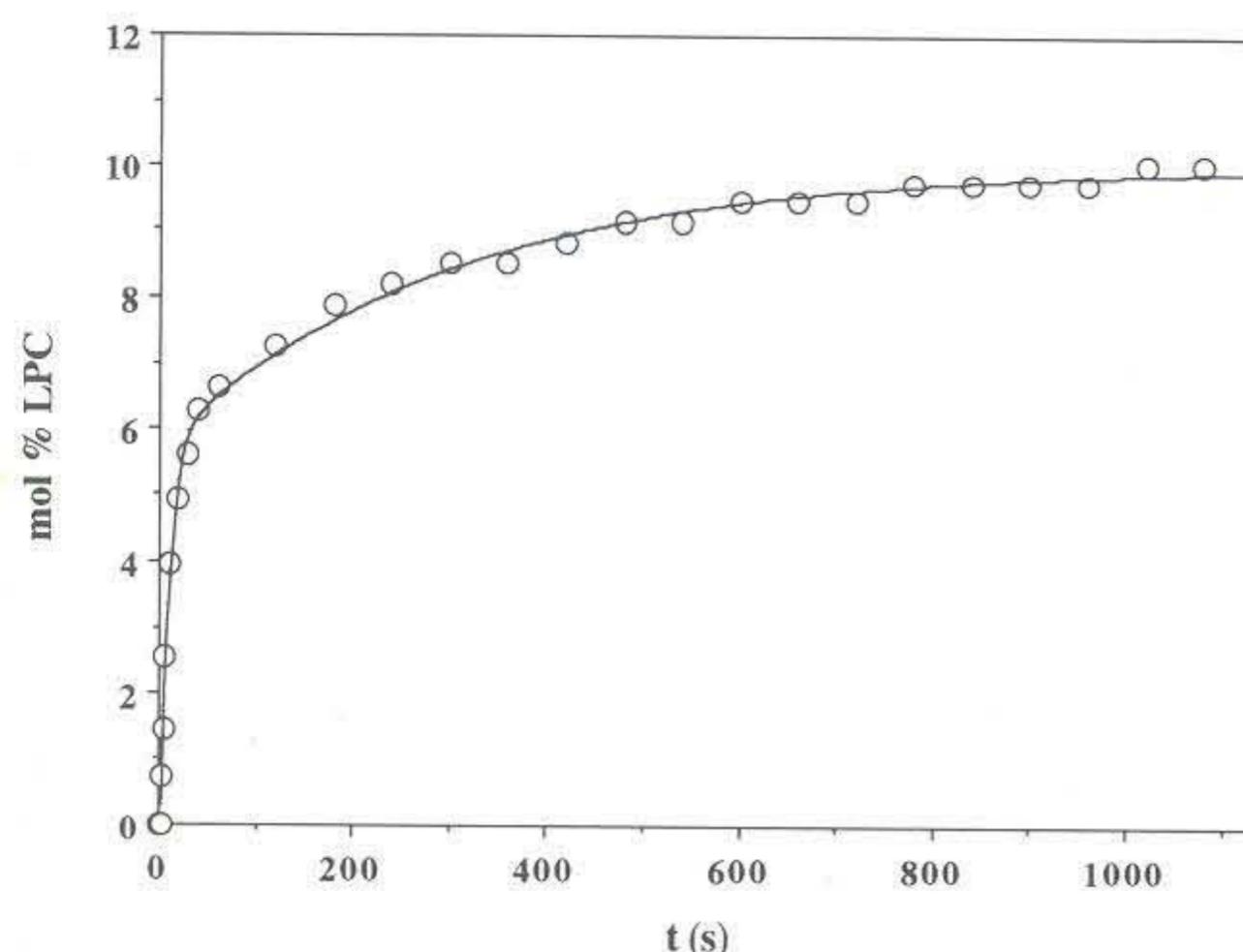
the above high levels of bilayer concentration, lysolipid must cross the bilayer midplane so that both outer and inner monolayers can attain equilibrium. In so doing, we expect that the rate of transfer across the bilayer midplane will be accessible to measurement.

Lysolipid uptake was therefore measured for extended time intervals ( $\sim 30$  min) using the flow method in an attempt to reach true equilibrium as predicted by the PC/LPC phase diagram. The experiment was carried out at low membrane tensions ( $\sim 0.1$ – $0.2$  dyn/cm), because we have found recently that the intercalation of lysoPC reduces the membrane strength (McIntosh *et al.*, submitted for publication). This induced fragility was also observed for other micellar surfactants, such as bile salts (20). The result, shown in Fig. 9, was that when subjected to flow of a 5-μM lysolecithin solution, the vesicle membrane area increased by 21% before the vesicle broke. This area increase at failure corresponded to 24 mol% lysoPC uptake in the membrane. The extrapolated value of the apparent asymptote gave a maximum molar concentration for lysolecithin in the membrane of 30 mol%. It is expected that this maximum uptake will be even higher for higher concentrations of the surfactant. Nevertheless, this maximum value of  $\sim 30$  mol%, measured in eggPC bilayers was still a substantial uptake and approached the 50 mol% solubility limit for monooleoyllysolecithin in dioleoylphosphatidylcholine (53). In this experiment then, we were actually reaching the kinds of concentrations that are expected from the equilibrium phase diagram for a single bilayer vesicle.

As shown in Fig. 9, for these extended time scales and



**FIGURE 9.** Long time exposure of a single vesicle to a flow of 5-μM solution of lysolecithin. The experimental point are fitted with two exponentials with apparent rates of  $0.19 \text{ sec}^{-1}$  and  $0.0019 \text{ sec}^{-1}$ . The saturation limit of the membrane found from extrapolating the experimental point to infinity is 30 mol%. The arrow indicates where the first exponential crosses over to the second one (i.e., where the interbilayer transfer of lysolecithin becomes prevalent).



**FIGURE 10.** Long time exposure of red blood cell to flow of 10- $\mu\text{M}$  lysolecithin solution. The kinetics is a double exponential, corresponding to a fast saturation of the outside monolayer and a slower lysolipid transfer into the inside monolayer. The molar concentration in the membrane is an apparent value, assuming that the cell membrane is made only of lecithin. Because approximately half of the membrane is occupied by proteins, the true molar concentration in the lipid phase is  $\sim 20$  mol%.

higher LPC concentrations, the experimental data were fitted with two exponentials with fast and slow components. The first exponential dominated for the first 50 sec, while the second one fitted the experimental data for longer periods of time. As discussed earlier, in the initial stages lysolipid adsorbed and intercalated into the membrane at a rate that was dominated by diffusion across the stationary layer, and eventually reached an apparent saturation of 8 to 9%. LPC then continued to be taken up but with a much slower apparent rate. This slow component was determined by the rate of lysolipid transfer across the bilayer midplane and so gave an order of magnitude approximation for the transport of the phosphocholine headgroup from the outer monolayer to the inner monolayer down a surface density gradient. The rate constant for this intrabilayer transfer of  $0.0019 \text{ sec}^{-1}$  converts to a half-time of  $\sim 300$  sec. For comparison, this value is of the same order as pH gradient measurements made on phosphatidylglycerol ( $\sim 500$ – $1000$  sec) (45), is approximately two orders of magnitude faster than equilibrium flip-flop rates measured for phosphatidylethanolamine (9 hr) (22,54,55) and several orders of magnitude faster than equilibrium exchange rates measured for fluorescently labeled lipids (22) (3 to 14 days for phosphocholine). Taken together, these results show the importance of the chemical composition of the surfactant headgroup and density gradients in determining the rate of transfer across the bilayer midplane.

One of the motivations for our studies is that lysolecithin and other surfactants are expected also to be taken up

by biological membranes and this might have useful applications in gene transfection (see below). To test this and to make a comparison with the lipid bilayer studies, flow experiments were carried out on erythrocytes. Figure 10 shows the uptake of lysolecithin (flow of a 10- $\mu\text{M}$  lysolecithin solution) into the red cell membrane. Once again, both fast and slow uptake regimes were observed. The fast and slow exponentials had rates  $\sim 0.13 \text{ sec}^{-1}$  and  $0.003 \text{ sec}^{-1}$ , respectively, which were on the same order as the rates measured for eggPC bilayers. As with the lipid bilayer, the above results suggest that the initial intercalation of lysolipid occurs preferentially in the outside monolayer after which it is transferred into the inside monolayer by a much slower process.

#### POROUS DEFECTS IN MEMBRANES CONTAINING LYSOLIPID

The formation and evolution of pores in membranes is an important determinant for the delivery of membrane-insoluble molecules into the internal compartment of vesicles and cells. With the emergence of molecular biology as a tool for understanding cellular processes, (especially those relating to oncogenesis [8], the current focus on rational drug design [4], and the genetic manipulation of the immune system to fight cancer and AIDS [3,47]), there has come the need to deliver a range of molecules to intracellular sites, both *in vitro* and *in vivo*. Reliable methods are therefore required in order to perturb reversibly lipid and cell membranes for the specific purpose of introducing hydrophilic macromolecules, such as DNA and drugs, into cells under study or treatment.

At high concentrations of lysolecithin, we have found in other experiments (Zhelev and Needham, unpublished results) that the permeability of the membrane can be dramatically reduced while the membrane itself supports a far-field tension. This permeability appears to be in the form of reversible nanometer-sized pores. Thus, as we have begun to investigate, the composition, structure, and properties of the lipid bilayer membrane itself can be manipulated by introducing molecules such as lysolecithin. The presence of lysolecithin in lipid membranes is expected to influence pore formation and stability through changes in pore edge energy (57), and these changes should be manifest in the response of membranes to electroporation.<sup>5</sup> The issues addressed in this paper, then, are likely to be important in the design of protocols that promote the formation of membrane pores and promote higher yields for the addition of large molecules, such as genetic material, to the cellular cytoplasm.

<sup>5</sup> Electroporation is the formation of pores in cell membranes by high voltage electric shock (44).

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