Chemical Control of Phospholipid Distribution Across Bilayer Membranes

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Abstract: Most biological membranes possess an asymmetric transbilayer distribution of phospholipids. Endogenous enzymes expend energy to maintain the arrangement by promoting the rate of phospholipid translocation, or flip-flop. Researchers have discovered ways to modify this distribution through the use of chemicals. This review presents a critical analysis of the phospholipid asymmetry data in the literature followed by a brief overview of the maintenance and physiological consequences of phospholipid asymmetry, and finishes with a list of chemical ways to alter phospholipid distribution by enhancement of flip-flop. © 2002 Wiley Periodicals, Inc. Med Res Rev, 22, No. 3, 251–281, 2002; Published online in Wiley InterScience (www.interscience.wiley.com). DOI 10.1002/med.10009

Key words: phospholipid asymmetry; flip-flop; erythrocyte membrane; phospholipid translocation

1. INTRODUCTION

The translocation (or flip-flop) of phospholipids across an abiotic bilayer membrane is known to be a very slow process with a half-life of hours to days (Fig. 1).[1–3] Flip-flop rates in these artificial systems are strongly dependent on the composition of the polar head-group, and less dependent on the length of the acyl chains.[4] In biological plasma membranes, however, transbilayer movement is significantly more facile for phospholipids with certain head-groups (refer to Scheme 1 for structures of the biological head-groups). For example, PE and PS have half-lives for inward translocation (flip) of only 5 min in the human erythrocyte, while PC and SM are relatively immobile.[5] It is reasonably well-established that the accelerated flip of the amino-containing phospholipids is due to membrane-bound aminophospholipid flippase enzymes.[6] Outward translocation (flop) in certain biological membranes also appears to be accelerated, and specific energy dependent proteins are believed to mediate this process.[7,8] Likewise, evidence exists for

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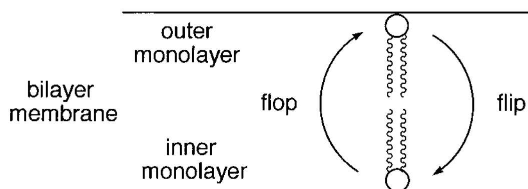
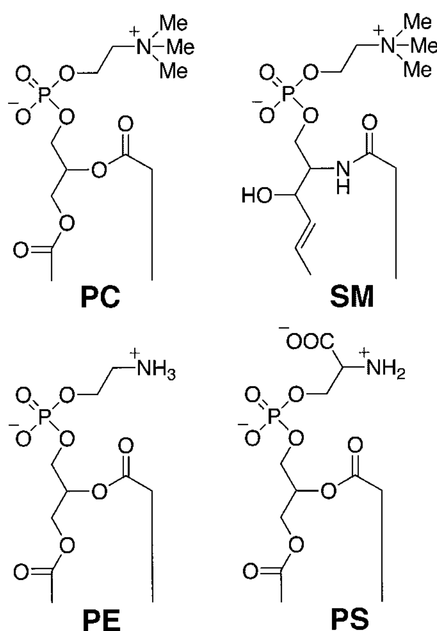


Figure 1. Schematic representation of phospholipid translocation or flip-flop across a bilayer.

bidirectional, nonspecific, nonenergy dependent, protein mediated flip-flop in the membrane of the endoplasmic reticulum,[9–14] mitochondria,[15] and several strains of bacteria.[16–18]

As discussed below, a range of chemical and biochemical assays have been developed to measure phospholipid distributions and rates of membrane translocation. The fragility of bilayer membranes makes these measurements technically challenging and in some cases it is difficult to get highly reproducible results. Thus it is not surprising that the quantitative data from different laboratories are sometimes in disagreement. However, there is now a consensus that the distribution of phospholipids across most if not all eukaryote and prokaryote membranes, as well as many viruses derived from eukaryotic host cells, is not symmetric. The degree of asymmetry varies among the different types of membranes. Plasma membranes of most eukaryotes maintain a high degree of asymmetry. A lesser degree of transbilayer asymmetry is observed in the membranes of subcellular organelles and bacterial species, which may reflect the higher, nonspecific endogenous phospholipid translocation rates in these systems.

Concurrent with the recent increase in knowledge of the mechanisms behind signal transduction is an increased understanding of the reasons why a cell expends energy to maintain an asymmetric distribution of phospholipids. From a medicinal perspective, researchers are beginning to appreciate the physiological consequences of using chemicals to alter phospholipid distributions, and hopefully in the future these chemical effects can be harnessed and developed into useful pharmacological strategies or tools for biomembrane research.



Scheme 1. Common phospholipid head-group structures.

2. METHODS TO MEASURE PHOSPHOLIPID DISTRIBUTION AND FLIP-FLOP

Several methods have been established for measuring both transbilayer distribution and flip-flop rates. Reagents used in these techniques must meet several requirements. First, they must only interact with the lipids of the outer monolayer and not permeate the membrane. Second, the reagents should not alter or scramble the bilayer distribution. It is also vital that endogenous lipid translocation rates be low throughout the course of the measurement. The most widely used methods for determining asymmetry of endogenous phospholipids include chemical modification, enzymatic degradation by phospholipases, and phospholipid exchange. Chemical modification is based on the availability of a reactive amine group, therefore, it can only detect the transbilayer distribution of PS or PE. The most commonly used reagent is trinitrobenzenesulfonate (TNBS),[19–22] but others such as formyl-methionyl sulfone methyl phosphate (FMMP)[23–25] and isethionyl acetimidate (IAI)[26] have been used. The most challenging aspect of this method is establishing experimental conditions in which the probe does not gain access to the lipids in the inner monolayer. Permeability is not a problem when using phospholipases[27–29] and phospholipid exchange proteins[2,3] because these proteins are too large to pass through the membrane. Phospholipases, however, have to be used under nonlytic conditions. Lysis is often a problem because the products of enzymatic degradation, lyso derivatives and fatty acids, are known to destabilize bilayers. Phospholipid exchange proteins are thought to be less destructive to membrane structure, however, the use of both enzymatic degradation and exchange proteins requires long incubation times.

While phospholipases and phospholipid exchange proteins can be employed to determine resting state asymmetry, the long incubations mean these methods cannot be employed to determine flip-flop rates. The use of synthetic reporter lipids with a shortened acyl chain at the C2-position has greatly facilitated these types of studies. Having one short acyl chain renders these lipids slightly water soluble, allowing for facile incorporation into the outer monolayer of both vesicles and cells. The shortened acyl chain bears a reporter group such as a doxyl spin-label[5] or a fluorescent 7-nitrobenz-2-oxa-1,3-diazol-4-yl (NBD) dye.[30,31] At specific time intervals after addition of flip catalysts, the adjusted lipid distribution is revealed upon chemical reduction of the spin-label or NBD group by the nonpenetrating agents ascorbate or dithionite, respectively. It is also possible to back extract the reporter lipid remaining in the outer monolayer using albumin. Recently, a new fluorescent reporter lipid was characterized with a pyrene derivative on its short acyl chain.[32] Transbilayer distribution is easily monitored because the ratio of the fluorescent intensities of the eximer and monomer pyrene bands depends upon the concentration of the probe in the monolayer, however problems may be encountered due to the large size of the dye. The general drawback of using reporter lipids is that there is no guarantee that they behave exactly like endogenous lipids.[33,34]

Transbilayer diffusion of lipids can also be monitored in red blood cells and platelets through morphology assays.[35,36] According to the bilayer couple hypothesis, exogenously added amphipath initially incorporates into the external monolayer of cells, converting the normal discocyte shape to an echinocyte characterized by spicules. If the added lipid redistributes into the inner monolayer over time, cup-shaped stomatocytes will form.[37]

Several noninvasive techniques have been devised to specifically measure endogenous PS (and other anionic lipids) distribution in a normal or chemically altered cell. These methods are based on protein binding properties. The low-molecular-weight protein, annexin V, has an extremely high affinity for anionic phospholipids.[38,39] Tagged with a fluorophore, the amount of protein bound to a cell or vesicle can be easily monitored via flow cytometry. Another method for monitoring PS exposure is the prothrombinase assay.[40–43] Anionic lipids serve as a binding site for the prothrombinase complex. Successful complex formation results in the generation of thrombin, which may be monitored spectroscopically. The activation of exogenous protein kinase C can also provide a sensitive measure of the amount of PS present in the outer monolayer of membranes.[44]

3. CRITICAL ANALYSIS OF PHOSPHOLIPID DISTRIBUTION DATA

A variety of cell membranes have been analyzed for phospholipid distribution between the two monolayers. The bulk of this data was collected in the 1970s and 1980s, however, work continues and reports on previously uncharacterized cells still appear. All of the results in the following discussion have been tabulated in Table I.

A. Erythrocyte Membrane

1. Human Erythrocyte Membrane

In 1971, Bretscher first hypothesized that the phospholipids of the human erythrocyte membrane were arranged asymmetrically.[23] He used the nonpenetrating probe, [^{35}S]FMMP, to label free amino groups of membrane proteins and noticed that negligible amounts of PE and PS were labeled in intact erythrocytes. However, significantly more labeling occurred in unsealed ghosts.[24] In follow up studies Bretscher determined that the results were not affected by protease treatment, and therefore, a PE-protein interaction was not preventing the lipid from being labeled with FMMP.[25] He concluded that 34- and 16.5-times as much PE must be in the inner monolayer compared to the outer monolayer of intact cells and ghosts, respectively. However, he employed a very small concentration of probe, meaning that he only studied the initial rates of the labeling reaction. Yet, Gordesky et al. verified the proposed asymmetry with a different chemical probe, TNBS.[20,21] This apparently nonpenetrating probe labeled 33% of the PE over a 24 hr period; no labeling of PS occurred. Performing the same experiment on unsealed erythrocyte ghosts resulted in 95% of PE and 50% of PS being labeled. As with FMMP, labeling was not affected by protease treatment.

There is controversy in the literature concerning the validity of TNBS as a nonpenetrating probe. Similar to the study described above, Bonsall and Hunt claimed that human erythrocyte membranes are impermeable to TNBS,[19] however, Arrotti and Garvin discovered that TNBS was passing through the membrane under their experimental conditions of phosphate buffer at 37°C.[45] Gordesky et al. also observed probe penetration under these conditions, therefore, they claimed the ideal condition to be 23°C in a bicarbonate buffer.[21] However, even at the low temperature of 15°C, the membrane of *Bacillus megaterium* is permeable to TNBS.[46] These studies highlight the importance of establishing appropriate conditions for each experiment. Typically, researchers demonstrate that TNBS does not significantly react with the amino groups of hemoglobin under their experimental protocol. Observing plateaus in the amount of reacted TNBS over time is also indicative of nonpenetrating conditions. Then, upon permeabilization of the membrane, both the reaction rate and the amount of labeled hemoglobin should increase. Haest et al. claim that a more sensitive method of detecting TNBS penetration is to monitor the decrease in glutathione content.[22] The reaction of TNBS with SH groups occurs before covalent binding to amino groups, so the large amount of SH groups present in the erythrocyte membrane may act as a sink for the probe. For example, glutathione levels decreased 40% within 15 min at 37°C. However, under similar conditions to those used by Gordesky et al.,[21] Haest and Deuticke blocked glutathione with the presence of SH reagents and still were unable to observe TNBS penetration,[47] lending validity to the asymmetric distribution claimed by the former.

Because of the conflicting opinions concerning the use of TNBS, Whiteley and Berg developed an alternate probe, IAI.[26] This nonpenetrating probe covalently reacts with outer monolayer amino groups under milder physiological conditions than employed for TNBS. Using radiolabeled IAI, only 0.3% of lipid amino groups were labeled in human erythrocyte membranes. They concluded that 100 times as many reactive amino groups reside in the inner monolayer.

Because experiments with amino-labeling reagents suggested that the amino-containing phospholipids were localized in the inner monolayer, it was assumed that the choline-containing

Table I. Asymmetric Phospholipid Distributions Determined for Several Biological Membranes

Type of membrane	*Percentage of each phospholipid present in the outer monolayer*							
	SM	*PC*	*PE*	*PS*	*PI*	*CL*	*Other*	*References*
Eukaryotic plasma membranes								
Human erythrocyte			33	0				20
	83	62						27
	85	68						28
			15	0				21
		75						49
					20		20[a]	53, 54
Rat erythrocyte	100	62	20	6[b]				57
Monkey erythrocyte		70	18					58
Mouse erythrocyte	85	57	20	0	42			59
Human platelet								
Resting state	93	45	20	9	16			91
		38	30	3	20			92
Activated state		45	30	18	34			92
Mouse LM		48	30					82
Mouse erythroleukemic cell	80	45	47	14				59
Chick embryo fibroblasts			35	20				83
Chick embryo myoblasts			65	45				83
Quail embryo myoblasts			63	44				83
S. carlsbergenis yeast			15	10	15			93
Cultured cells								
MDBK cells[c]	19	43	29	24	80			75
BHK-21 cells	83	53	35	< 5				81
(VSV)[d]			36					76
	80	94	43					77
	64							78
(SFV)[e]	33	50	22					79
	95	55	20	< 5				80
Schwann cells	90	89	15	4	31			84
Eukaryotic intracellular membranes								
Rabbit muscle sarcoplasmic reticulum			70	0				73
Endoplasmic reticulum								
Castor bean endosperm		55	80		0			74
Smooth rat liver	63	76	40	12				63
Rough rat liver	58	68	40	26				63
Inner mitochondria								
Beef heart		73	39			40		64
Rat liver			61					66
		54	91		15	18		67
Outer mitochondria								
Rat liver		55	77	30[b]		100		69
Cholinergic synaptic vesicles								
N. brasiliensis			62	40				70
T. ocellata		58	77	0	100		47[f]	71
N. japonica		86	59	69				72

(*Continued on next page*)

Table I. (*Continued*)

Type of membrane	Percentage of each phospholipid present in the outer monolayer							References
	SM	PC	PE	PS	PI	CL	Other	
Prokaryotic plasma membranes								
M. lsodeikticus					20	50	80[g]	85
B. subtilis			60				60[h]	86
B. amyloliquefaciens			90			30	90[g]	87
B. megaterium			33					46

[a] Both PIP_2 and PA (PIP found in outer monolayer).
[b] Amount of PS and PI combined.
[c] Distribution determined from influenza virions budded through MDCK host cell.
[d] Distribution determined from VSV virions budded through BHK-21 cells.
[e] Distribution determined from SFV virions budded through BHK-21 cells.
[f] Plasmenylethanolamine.
[g] PG.
[h] Lysyl PG.

phospholipids were located on the exterior surface. Direct evidence for external exposure of PC and SM was obtained using phospholipases. Digestion of intact erythrocytes with phospholipase A_2 from *Naja naja* venom resulted in 68% PC hydrolysis, while digestion with sphingomyelinase degraded 85% of the SM.[27,28] These large enzymes are unable to permeate the membrane of intact cells under nonlytic conditions, consequently they only react with lipids in the outer monolayer. In unsealed erythrocyte ghosts, however, they act on both sides of the membrane. In this latter case, digestion with the two enzymes described above resulted in complete degradation of both phospholipids. Likewise, phospholipase A_2 and C were used to provide direct evidence for the internal localization of PE and PS, as opposed to basing conclusions on negative labeling results.[48] Enzymatic digestion of sealed inside-out erythrocyte membrane vesicles degraded nearly all of the PS and PE and only 30–40% of the PC and SM. Pancreatic phospholipase A_2 was also used to directly prove the inner monolayer localization of PE and PS in erythrocyte ghosts.[49] This enzyme does not hydrolyze any lipid when added externally to cells. However, it can be trapped inside resealed ghosts in an inactive state with its Ca^{2+} cofactor chelated by EDTA. Upon addition of Ca^{2+}, 25% of the PC, 50% of the PE, and 65% of the PS were hydrolyzed before cell lysis became a problem. It is of interest to note that PC hydrolysis had reached a plateau at this point, while PE and PS were still being hydrolyzed.

The asymmetric state of the erythrocyte membrane was more or less accepted by the late 1970s, however, several criticisms were still brought forth.[50] Aside from the debates concerning chemical probe permeation, it was also argued that the production of nonbilayer forming lipids during phospholipase treatment was altering the phospholipid distribution. Furthermore, many direct PS and PE localization experiments employed ghosts, resealed ghosts, or inside-out vesicles. Clearly, preparation of these bilayers could alter membrane distribution.

The question concerning the asymmetry perturbation in resealed erythrocyte ghosts was partly answered in 1985.[51] Asymmetry was retained when ghosts were prepared in the presence of Mg^{2+} as the only divalent cation. However, the presence of Ca^{2+} (concentrations as low as 10 μM) resulted in scrambled membranes. These results were later verified by Schrier et al. who generated asymmetric ghosts upon resealing in the presence of Mg-ATP.[52]

The asymmetry of the phosphoinositides (PI, PIP, PIP_2) and PA has also been determined for the human erythrocyte. Using a PI-specific phospholipase C, Bütikofer et al. degraded 24% of the PI.[53] They confirmed their result by extracting 18% of the total PI with albumin. Similar results were reported by Gascard et al.[54,55] Based on phospholipase A_2 hydrolysis, they claimed ~20% of both the PIP_2 and the PA is present in the outer monolayer, while virtually all of the PIP resides in the

inner monolayer. In both of the studies, the results were verified using PIP and PIP_2 antibody staining. The phosphoinositides, however, represent a very small percentage of the total phospholipid content of the erythrocyte membrane.

2. Other Erythrocyte Membranes

Once the methods of determining asymmetry were established through extensive study of the human red blood cell, many other membranes were investigated in rapid succession. The phospholipid distribution of rat erythrocytes was examined in both ghosts and intact cells. Approximately 75% of the PC in ^{32}P-labeled resealed ghosts was available for rapid exchange by the phospholipid exchange protein,[56] while enzymatic degradation of intact cells by phospholipase A_2 and sphingomyelinase led to a proposed distribution that is very similar to human erythrocytes.[57] The outer monolayer is believed to contain 100% of the SM, 62% of the PC, 20% of the PE, and 6% of the PS/PI.

Similarly, the membranes of monkey red blood cells also have an asymmetric distribution that is analogous to the human membrane.[58] Roughly 70% of the PC was hydrolyzed in the presence of phospholipase A_2, while 15–20% of the PE was labeled with TNBS. Because no PS was hydrolyzed or labeled, it was postulated to reside in the inner monolayer. SM, by default, was placed in the outer monolayer. Interestingly, upon infection of monkey erythrocytes with the malaria parasite, a change in asymmetry is induced.[58] Only $\sim$25% of the PC remains in the outer monolayer, while the percentage of PE in this layer increases to $\sim$50%.

Enzymatic degradation of mouse erythrocytes by sphingomyelinase and phospholipase A_2 under nonlytic conditions hydrolyzed 85% of the SM, 57% of the PC, 20% of the PE, 42% of the PI, and no PS.[59] With the exception of a decreased amount of PC and increased amount of PI, this distribution is also similar to that of the human erythrocyte. The distribution of a mouse erythroleukemic cell (erythroid cell blocked at early stage of differentiation) is similar, however, the monolayer concentrations of PC, PE, and PS are altered slightly compared to the mature erythrocyte.[59]

B. Intracellular Membranes

Investigations of subcellular membranes were conducted to determine the generality of the asymmetric membrane phenomenon, however, several contradictory results exist in the literature. The first report of an asymmetric distribution of the endoplasmic reticulum membrane appeared in 1977 by Nilsson and Dallner.[60] Phospholipase A_2 (*Naja naja*) digestion of rat liver microsomes in the presence of albumin was reported to be nonlytic. The authors concluded that PC was symmetrically distributed and that the majority of the PE and the PS was in the outer (cytoplasmic) monolayer, whereas the majority of the PI was localized in the inner (lumenal) monolayer. Later in the same year, however, a contradictory report by Sundler et al. claimed a symmetric distribution of PC, PE, and PI in rat liver microsomes.[61] Their results were based on labeling with IAI and digestion with phospholipase A_2 (*Naja naja*). It is interesting to note that they did not use albumin in their digestion protocol (Nilsson and Dallner reported that its presence was necessary to avoid lysis[60]). Further contradiction was encountered when Higgins and Dawson reported that phospholipase A_2 from both bee venom and *Naja naja* resulted in 80% lysis of microsomes, and the addition of albumin only increased the release rate.[62] They claimed that phospholipase C from *Clostridium welchii* treatment was nonlytic under their conditions. Their hydrolysis results suggest that 70% of the SM, 72% of the PC, 18% of the PE, and 16% of the PS is located in the outer monolayer. Nearly all of the PI was assigned to the inner monolayer. Bollen and Higgins refined their study of microsomal membranes by reporting the asymmetries associated with both the smooth and rough ER (all of the earlier results were obtained for total ER membranes).[63] The outer monolayer of

smooth ER membranes was reported to contain 63% of the SM, 76% of the PC, 40% of the PE, and 12% of the PS; while rough ER outer monolayers consisted of 58% of the SM, 68% of the PC, 40% of the PE, and 26% of the PS. Strikingly, these results are nearly opposite to those reported by Nilsson and Dallner.[60] However, a separate study revealed that the phospholipase A_2 preparation used in both Nilsson and Dallner's work,[60] and that of Sundler et al.,[61] was most likely contaminated with a small lytic peptide that binds tightly to the phospholipase.[64]

The asymmetric arrangement of inner mitochondrial membranes has also been investigated in various organisms, again yielding contradictory results.[65] An early study indicated that 60–65% of PE was labeled by TNBS in right-side-out mitoplasts from rat liver mitochondria, suggesting that it is localized in the outer monolayer.[66] Based on hydrolysis results with phospholipase A_2 (*Naja naja*), Nilsson and Dallner also claimed that the majority of PE was localized in the outer monolayer, while the bulk of the PI and the CL resided in the inner monolayer.[67] PC was suggested to be symmetrically distributed. However, as noted above, their enzyme preparation may have failed to remove a lytic peptide.[64] In contrast, a study performed on beef heart right-side-out mitoplasts and inside-out submitochondrial particles revealed that 38 and 63%, respectively, of the PE was labeled with the nonpenetrating probe fluorescamine.[64] Along with PE, the majority of the CL was determined to be in the inner monolayer based on antibody staining. A high percentage of the PC was claimed to reside in the outer monolayer.

Phospholipid distribution in the outer mitochondrial membrane has been reported for a couple of organisms. The outer membrane of yeast (*Saccharomyces cerevisiae*) was subjected to PC and PI transfer protein analysis and TNBS labeling.[68] Both PC and PI were found to be evenly distributed, while 80% of the PE was localized in the inner monolayer. More recently, the outer mitochondrial membrane of rat liver was analyzed via phospholipase A_2 digestion, PC transfer protein treatment, and TNBS labeling to reveal an outer monolayer composition of 55% of the PC, 77% of the PE, 30% of the PS/PI, and 100% of the CL.[69]

Cholinergic synaptic vesicles from several marine rays have been characterized. Reaction of synaptic vesicles from *Narcine brasiliensis* with IAI labeled 62% of the PE and 40% of the PS in the outer (cytoplasmic) monolayer.[70] The presence of these fusogenic lipids on the cytoplasmic face is likely to promote intracellular fusion events. Likewise, results obtained from phospholipase C digestion of *Torpedo ocellata* cholinergic synaptic vesicle membranes suggest that the outer monolayer contains 58% of the PC, 77% of the PE, 100% of the PI, and 47% of the plasmenylethanolamine.[71] It is presumed that all of the PS is localized in the inner monolayer based on the absence of hydrolysis, however, this lipid is a poor substrate for the phospholipase. Following the equilibrium distributions of NBD-lipid derivatives, the inner monolayer of *Narke japonica* synaptic vesicles membranes is postulated to contain 14% of the PC, 41% of the PE, and 31% of the PS.[72] Again, a significant amount of fusogenic PE and PS resides in the outer monolayer.

Another example of an asymmetric distribution in a subcellular membrane is the sarcoplasmic reticulum membrane isolated from rabbit white skeletal muscle.[73] A TNBS labeling study revealed that the majority of the PE is located externally, while nearly all of the PS is in the inner monolayer. Finally, digestion of rough endoplasmic reticulum of castor bean endosperm by phospholipase A_2 suggests that 55% of the PC and 80% of the PE is present in the outer monolayer, whereas close to 100% of the PI is restricted to the inner monolayer.[74] This study extends the scope of asymmetric membranes to plants.

C. Complex Eukaryotic Plasma Cell Membranes

The assessment of plasma membrane phospholipid distribution is significantly more difficult in higher eukaryotes as compared to erythrocytes. Isolating pure plasma membrane samples in the form of sealed vesicles is problematic due to intracellular membrane contamination. Two common

approaches to circumvent the problem are: (1) studying virions from budding viruses that obtain their lipid membrane from the host cell plasma membrane in a right-side-out manner, or (2) studying inside-out vesicles obtained from the uptake of inert latex particles (phagocytic vesicles).

1. *Budding Viruses*

Rothman et al. used the first approach to study the asymmetry of the influenza virus grown on Maden Darby bovine kidney cells (MDBK).[75] Phospholipid exchange protein and phospholipase C treatment revealed that only ~30% of the total phospholipid was accessible to the probes on the outer monolayer surface, likely due to the high glycolipid content of virus grown in MDBK cells. The transbilayer distribution of the accessible PE and PS was roughly symmetrical. However, PC and PI were enriched in the outer monolayer, whereas SM was enriched in the inner monolayer.

In principle, the asymmetrical distribution determined for different viruses budded from the same cell line should be similar. This appears to be the case for vesicular stomatitis virus (VSV) and Semliki Forest virus (SFV) grown on baby hamster kidney cells (BHK-21).[76–80] An asymmetric distribution of PE (36% in outer monolayer, 64% in inner monolayer) was found for VSV using the impermeable TNBS reagent.[76] In good agreement, another laboratory reported 38% labeling of the PE with TNBS, while phospholipase C hydrolyzed 80% of the SM, 94% of the PC, and 47% of the PE.[77] Because PS is not a good substrate for this enzyme, they could only postulate that it was localized in the interior monolayer. A later study with the PC exchange protein suggested a slightly decreased amount of the PC in the outer monolayer.[78]

Similarly, SFV grown on BHK-21 cells has a low distribution of PE (22%) in the outer monolayer.[79] Approximately 33% of the SM and 52% of the PC comprised the remainder of the outer monolayer. Nearly identical percentages of external PC and PE were reported 8 years later by Allan and Quinn.[80] In addition, they determined that $<5\%$ of the PS was located externally. However, 95% of the SM was found to be in the outer monolayer in this study. They claim that the earlier study[79] used suboptimal conditions for sphingomyelinase hydrolysis. These authors also claim that the low amount of SM reported to be in the outer membrane of the influenza virus (grown on MDBK cells) by Rothman et al.[75] was an artifact of using phospholipase C which does not specifically attack SM. Whatmore and Allan later analyzed plasma membrane vesicles derived directly from BHK-21 cells.[81] Degradation by phospholipase A_2 and sphingomyelinase verified the phospholipid distribution determined for SFV.

The consensus of the phospholipid distribution data for plasma membrane vesicles originating from BHK-21 cells (see Table I) is that the asymmetry is very similar to that of the human erythrocyte with the exception of a more symmetrical arrangement of PC. Because the majority of these results were derived from virions, it is important to consider that the budding process may only occur at specialized regions of the host plasma membrane.[79,80]

2. *Phagocytic Vesicles*

The distribution of PC and PE in inside-out phagocytic vesicles from mouse LM cells was analyzed using a PC transfer protein and TNBS.[82] Approximately half of the PC was available for exchange and 70% of the PE was chemically labeled, implying a symmetric distribution of PC whereas 70% of the PE resides in the inner monolayer. The authors speculate that SM resides primarily in the outer monolayer. Phospholipid distribution in chick embryo fibroblasts and myoblasts was investigated via TNBS and IAI reaction with both inside-out phagocytic vesicles and right-side-out vesicles obtained from chemically induced membrane blebs.[83] The two methods complemented each other, revealing that the outer monolayer of fibroblasts contains 35% of the PE and 20% of the PS, while that of the myoblast contains increased percentages of 65 and 45%, respectively. In the same study, the outer

monolayer of quail myoblasts was found to contain 63% of the PE and 44% of the PS. The higher percentage of amino-containing lipids in the outer monolayer may be required for myoblast fusion.

More recently, phospholipid asymmetry was reported for the Schwann cell line that is derived from a human neurofibroma.[84] Rather than utilizing either of the two methods discussed above, phospholipase degradation was performed directly on cells. Similar to erythrocytes, 90% of the SM and 89% of the PC is located in the outer monolayer. The inner monolayer is enriched in PE, PS, and PI (85, 96, 69%, respectively).

D. Bacterial Membranes

The existence of asymmetric plasma membranes is less certain in bacteria than in eukaryotes. Studies on the phospholipid distribution of gram-positive bacteria require removal of the cell wall via lysozyme digestion. The remaining protoplasmic membranes are subjected to compositional analysis. Phospholipase and phospholipid transfer protein treatment on both intact and protease-digested protoplasts of *Micrococcus lysodeikticus* revealed that PG resides chiefly in the outer monolayer, while PI is located internally.[85] CL was symmetrically distributed between both monolayers. Approximately 60% of the PE in *Bacillus subtilis* protoplasts was accessible for TNBS labeling, however 90% was hydrolyzed by phospholipase C under nonlytic conditions.[86] The higher concentration of lipid available for hydrolysis was explained by an increase in flip-flop rate during phospholipase C treatment. In *Bacillus megaterium* membranes, TNBS and IAI reaction suggested an inner monolayer localization of PE.[46] By default, the other major phospholipid, PG, was presumed to be concentrated in the outer monolayer. Intact protoplasts of *Bacillus amyloiquefaciens* were treated with phospholipases and TNBS to reveal that 90% of both the PE and PG are localized in the outer monolayer, while the majority of CL is in the inner monolayer.[87]

Studies on gram-negative bacteria such as *Escherichia coli* have not been as successful.[88] Researchers have been able to detect an asymmetric distribution between the two membranes, but not among individual monolayers. The outer membrane is enriched in PE, whereas the cytoplasmic membrane is enriched in PG and CL.[89]

E. Other Membranes

Several other membranes also possess transbilayer asymmetry. The outer monolayer of the lipid-containing bacteriophage PM2 consists mainly of PG; the inner monolayer is enriched in PE.[90] The outer monolayer composition of the human platelet plasma membrane was determined by phospholipase digestion. It contains 93% of the SM, 45% of the PC, 20% of the PE, 9% of the PS, and 16% of the PI.[91] Years later, use of the basic phospholipase A_2 verified the distribution in the resting state of the platelet: 38% of the PC, 30% of the PE, 3% of the PS, and 20% of the PI.[92] This study went further to probe the phospholipid distributions in the thrombin-activated and thrombin-induced shape-changed states. The shape-changed state asymmetry was similar to the resting state, however in the activated state, the amount of PS and PI in the outer monolayer increased to 18 and 34%, respectively. A marginal increase in PC was observed. An asymmetric phospholipid distribution was also determined for the membrane of *Saccharomyces carlsbergenis* yeast.[93] TNBS labeling and PI-specific phospholipase C treatment revealed that ~90% of the PS and ~85% of both the PE and the PI are localized in the inner monolayer. Presumably, the majority of the PC resides externally.

4. MAINTENANCE OF ASYMMETRY

As mentioned briefly in the introduction, proteins are thought to play an important role in maintaining the transbilayer distribution of phospholipids. Translocase enzymes are thought to exert their

influence by controlling the rates of phospholipid translocation. Others believe that specific interactions between lipids and the membrane cytoskeleton maintain the distribution. These two hypotheses are summarized below.

A. Role of Translocase Enzymes

Eukaryotic cells possess enzymes generally known as "translocases" that maintain membrane asymmetry by promoting phospholipid translocation. While there is good evidence in favor of the existence of these enzymes, mechanistic understanding at the molecular level is lacking. The translocases could be specifically interacting with lipid head-groups, or perhaps they generate pores or channels. Three classes of translocases are defined depending upon the direction of lipid transport: a flippase facilitates inward translocation, a floppase facilitates outward translocation, and a scramblase facilitates translocation in either direction (Fig. 2).[6,94,95]

The aminophospholipid translocase is an ATP-dependent flippase that catalyzes unidirectional transport of PS and PE from the outer to inner monolayer with a half-life of translocation of 5–10 min. For every molecule of ATP hydrolyzed, one lipid moves across the membrane.[96] The existence of this enzyme was first postulated in 1984 by Seigneuret and Devaux,[5] and since then several potential candidates have been isolated from both erythrocyte and organellar membranes. Schroit et al. purified the first candidate, a 32 kDa protein from erythrocyte membranes that is similar to proteins of the Rh blood group complex.[97,98] The protein is not likely an ATPase according to amino acid sequence,[99] and furthermore, Rh_{null} cells have demonstrated normal PS transport.[100,101] However, it could be a structural or regulatory component of a larger aminophospholipid translocase complex. Another candidate is an erythrocyte Mg^{2+}-ATPase discovered in several laboratories. Morrot et al. isolated a partially purified sample containing a 116 kDa Mg^{2+}-ATPase.[102] Another partial purification conducted by Auland et al. revealed a 110 kDa ATPase,[103,104] while the Daleke group has repeatedly isolated an 80 kDa candidate.[105,106] Discrepancies between the molecular mass and specific activity of the three Mg^{2+}-ATPase candidates may be due to differences in experimental procedure.[6] Additionally, a 115 kDa protein, ATPase II, has been isolated from bovine chromaffin granules[107,108] and clathrin-coated vesicles[109] and shown to have similar properties to the human erythrocyte Mg^{2+}-ATPase. The bovine chromaffin granule protein has been cloned and partially sequenced, and it appears to be homologous to the P-type ATPases.[110] More specifically, the putative enzyme belongs to a subfamily, the Type IV ATPases.[111] The protein contains 10 transmembrane helices and 3 consensus sequences. The first consensus sequence is involved in the coupling of ATP hydrolysis to transport, the second contains an aspartate that is phosphorylated in the intermediate state, while the third is implicated in ATP binding. A homolog of the protein in

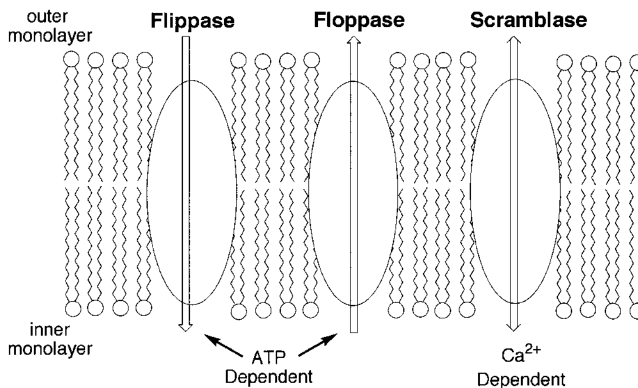


Figure 2. Translocase enzymes responsible for maintaining phospholipid asymmetry in biological membranes.

yeast, DRS2, has been shown to be essential for PS transport,[110] although there are conflicting results.[112,113] Homologs have also been discovered in a mouse teratocarcinoma cell line[114] and human skeletal muscle,[115] while four isoforms have been identified in bovine brain.[116] Most recently, a homolog, ALA1, has been identified in the Arabidopsis genome.[117] Further research is needed in order to unequivocally determine that this putative enzyme is responsible for flippase activity.

Another ATP-dependent enzyme, often referred to as a floppase, is responsible for moving lipids from the inner to outer monolayer with less head-group specificity and slower half-lives of translocation (~1.5 hr).[7,8] Two subfamilies of the ATP binding cassette (ABC) transporters have recently been reported to act as floppases.[118] These proteins are also known to participate in multidrug resistance. A member of the P-glycoprotein subfamily, MDR3, has been suggested to act as a specific PC translocator;[119,120] while the less specific MDR1 homolog transports short acyl chain lipid derivatives of PC, PE, and SM.[121,122] Outward translocation of endogenous lipid has been directly demonstrated with MDR3,[120] but never with MDR1. The crystal structure for a bacterial homolog of the MDR protein has been solved which may provide insight into the translocation mechanism.[123] A member of the multidrug resistance-related protein subfamily, MRP1, is also believed to be a nonspecific floppase capable of translocating PC, PS, and SM with similar rates.[124–126] Recently, Dekkers et al. obtained direct evidence of endogenous PC and SM flop induced by MRP1 using phospholipase A_2 and sphingomyelinase.[127]

The lipid asymmetry maintained by the flippase and floppase enzymes can be destroyed by an increase in the intracellular Ca^{2+} concentration.[128] This Ca^{2+}-induced scrambling is bidirectional and head-group nonspecific and has been attributed to an ATP-independent scramblase protein found in both erythrocytes and platelets.[129,130] The protein, PLSCR1, contains a conserved EF-hand related Ca^{2+}-binding motif which may undergo a large conformational change upon occupancy.[131,132] Other structural elements and mechanistic implications have been reviewed recently by Sims and Wiedmer.[133] To date, four homologs of the scramblase protein have been identified in humans.[134] An alternative hypothesis proposes that Ca^{2+}-induced scrambling is not a protein mediated process, but rather an effect of membrane alterations that result from PIP_2 complexed with Ca^{2+}.[55,135] This hypothesis has been refuted by others.[136]

Additional evidence in favor of the existence of translocase enzymes is that several inhibitors are known.[6] Aminophospholipid flippase activity is inhibited by cysteine- , histine- , and arginine-modifying reagents, thus indicating a potential role of these amino acids in lipid binding and translocation. An alternative approach is to inhibit the ATPase activity with vanadate or increased levels of Ca^{2+}. However, only one specific, competitive inhibitor of the flippase exists, glycerophosphoserine.[137]

B. Role of Cytoskeleton

It has been argued that selective interactions between the lipids of the inner monolayer and the proteins of the cytoskeleton play an important role in asymmetry maintenance. Haest et al. were the first to speculate that spectrin, one of the most abundant cytoskeletal proteins, was a key player in localizing PE and PS in the inner monolayer of human erythrocytes.[138] Other studies soon provided additional evidence for this hypothesis.[139,140] Preferential binding interactions between PS and spectrin,[141] as well as between PS and cytoskeletal protein 4.1,[142,143] provided even further support. On the other hand, there are extensive reports arguing against a cytoskeletal role in maintaining phospholipid distribution. Heating erythrocytes to 50°C, a condition known to induce irreversible structural alterations in spectrin, has no effect on bilayer asymmetry.[144–146] Additionally, erythrocyte vesicles with 75% less spectrin than normal cells were still capable of generating transbilayer asymmetry upon the introduction of exogenous aminophospholipids.[147] Furthermore, a radioactive photoactivable PE analog incorporated into both normal and symmetric erythrocyte ghosts was

crosslinked to cytoskeletal proteins with no distinguishable differences in the labeling patterns.[148] All of these results cast doubt on the necessity of cytoskeleton-lipid interactions for phospholipid asymmetry, although the existence of these interactions is not questioned. Several authors have postulated that a combined effect of translocase enzymes and cytoskeletal interactions is observed.[149–151]

5. CONSEQUENCES OF LOSS OF ASYMMETRY

The existence of an asymmetric transbilayer arrangement of phospholipids and the intricate energy consuming mechanisms that have evolved to maintain it imply that a loss in asymmetry has severe consequences.[152–154] For example, PS normally localized in the inner monolayer is vital not only for exocytosis and intracellular fusion processes, but also for lipid–protein interactions. A loss of asymmetry may decrease the number of PS molecules available for binding to protein kinase C, thus disrupting signal transduction pathways.[6] The role of phospholipid asymmetry in several signal transduction pathways is discussed in the recent review by Verkleij and Post.[155]

The appearance of PS in the outer monolayer of membranes correlates with cell death and clearance by phagocytes. It is well established that the external appearance of PS is a general feature in the induction of apoptosis,[156,157] and evidence exists to suggest that this mechanism is phylogenetically conserved.[158] Inactivation of the aminophospholipid flippase alone is not sufficient to expose PS; a simultaneous Ca^{2+} -dependent nonspecific scrambling event is necessary.[159] This loss of lipid asymmetry is believed to occur prior to DNA fragmentation, another characteristic apoptotic event.[160] PS-exposing apoptotic cells are cleared from the bloodstream by macrophages following a specific recognition event between the macrophage and the externalized PS.[161–165] Evidence for the specificity of the recognition lies in the ability of synthetic PS vesicles to inhibit erythrocyte clearance.[166] The absolute necessity of PS exposure was elegantly revealed in a recent study.[167] Apoptotic cells retaining their phospholipid asymmetry were generated, but these cells were not taken up by macrophages. Interestingly, a gene has been cloned that codes for a putative PS receptor on the surface of macrophages.[168]

Related to the apoptotic mechanism, it is believed that aging erythrocytes and platelets slowly externalize PS, culminating in engulfment by macrophages.[169–171] Aging erythrocytes also exhibit an increase in the amount of external PE.[172] Asymmetry may change with cell differentiation. For example, undifferentiated cells may have a lesser degree of asymmetry.[41]

One last consequence of lipid randomization is the regulation of hemostasis and thrombosis. The binding of activated platelets to proteins involved in the coagulation cascade is dependent on PS exposure.[40,173] The tenase and prothrombin complexes bind to the patches of anionic lipid on the cell surface, and through a series of activation steps the fibrin matrix of the clot is formed. There is also evidence that suggests the appearance of PS in the outer monolayer of erythrocytes invokes abnormal adherence to vascular endothelial cells.[174–176] This phenomenon is thought to play a role in the microvascular occlusions that characterize many disease states such as sickle cell, diabetes, thalassemia, and malaria. Increased PS exposure in erythrocytes is also believed to be associated with chronic renal failure.[177]

6. ALTERING ASYMMETRY BY FORMING TRANSIENT DEFECTS IN THE MEMBRANE

The cellular consequences for loss of phospholipid asymmetry are dramatic, thus it should be useful to develop chemical and physical treatments that promote flip-flop and experimentally manipulate the distribution. Broadly speaking, these treatments can be classified into two major groups that will

be discussed in the following sections—those that introduce transient defects into the membrane, and those that do not.

A. Anesthetics and Amphiphiles

1. Local Anesthetics

Local anesthetics are known to directly interact with both the lipid and protein components of membranes, often affecting membrane fluidity. In erythrocyte membranes, these compounds have also been found to alter transbilayer asymmetry. It was first reported in 1977 that the local anesthetics tetracaine (1 mM) and lidocaine (10 mM), along with the amphiphilic drug chlorpromazine (0.24–0.48 mM), increase the susceptibility of PE to hydrolysis by phospholipases C and A_2 in chicken erythrocytes.[178] Presumably, PE normally sequestered in the inner monolayer flips across the bilayer to the outer monolayer in the presence of the drugs. Similarly, Bradford and Marinetti discovered that 1 mM tetracaine increased the amount of PE labeled by TNBS in human erythrocytes from 9.2 to 14%,[179] while Deuticke, et al. realized that the flip rate of outer monolayer $[^{14}C]$lysoPC was enhanced 50 times in the presence of 3.6 mM tetracaine.[180] The appearance of 50% of the PE in the outer monolayer was monitored using phospholipase A_2 (2.5 mM tetracaine). Less than 10% of the PS was detected externally. Similar results were obtained with dibucaine at threefold lower concentrations. These results suggest that perturbation of the lipid domain by anesthetics increases the transbilayer mobility of the phospholipids, culminating in a loss of asymmetry.

Another local anesthetic, benzyl alcohol, was examined for its ability to catalyze the transbilayer diffusion of short-chain spin-labeled PC, PE, and PS inserted into the outer monolayer of human erythrocytes.[181] Using ESR spectroscopy to monitor phospholipid distribution, the initial inward translocation rates of PC, PE, and PS were observed to increase in the presence of 30 mM benzyl alcohol at 37°C. The PS and PE flip rates were not affected by an aminophospholipid flippase inhibitor. Apparently, the anesthetic is capable of enhancing the passive flux of all the phospholipids. This nonspecific increase in flip-flop is likely due to a perturbation of the lipid domain.

2. Amphiphilic Compounds

Several amphiphilic drugs also alter transbilayer phospholipid asymmetry. For example, treatment of human erythrocytes with chlorpromazine (0.3 mM), perazine (0.3 mM), prochlorperazine (0.2 mM), primaquine (5.0 mM), or *n*-decylamine (0.6 mM) increases the amount of outer monolayer PE hydrolysis from 1.6% to 35–45%.[182] In good agreement, 35% of the PE was labeled with TNBS in the presence of chlorpromazine and primaquine (14% labeled in untreated cells). No changes in the membrane distribution of PS and PC were detected in this study.

In contrast, a later study suggested that intercalation of amphiphiles into the membrane of human erythrocytes is accompanied by the formation of transient nonbilayer phases that result in a net rearrangement of all membrane lipids in order to maintain the bilayer barrier.[183] This bilayer scrambling event was directly studied by Devaux et al. using short-chain spin-labeled phospholipids in the presence of 0.5–1.0 mM chlorpromazine.[184] If the PE and PS probes were allowed to flip to the inner monolayer before addition of chlorpromazine, 10–15% of both probes rapidly appeared in the outer monolayer. Likewise, if chlorpromazine was added after the incorporation of spin-labeled PC and SM in the outer monolayer, ~10% of these probes immediately flipped inside. They concluded that initial scrambling results from transient bilayer perturbation. Four years later, they studied chlorpromazine-induced (1 mM) stomatocytosis and refined their scrambling hypothesis.[185] They claimed that the rapid scrambling event was followed by flipping of PE and PS back to the inner monolayer via the aminophospholipid flippase. Because there is no rapid method to flop PC

and SM back to the outer monolayer, stomatocytes are produced. More recently, however, Chen and Huestis performed a detailed study on chlorpromazine-induced stomatocytosis that contradicts these results.[43] Using lower concentrations of chlorpromazine (120 μM), they revealed that stomatocytes are formed long before any redistribution of [^{14}C]DLPC can be detected. The ability of chlorpromazine to partially scramble phospholipid asymmetry occurs over extended time periods.

Detergents are another class of amphiphilic compounds capable of disrupting bilayer asymmetry. Hägerstrand et al. investigated the ability of anionic and nonionic detergents to induce PS exposure in human erythrocytes as detected by annexin V binding.[186] At sub-lytic concentrations, octaethyleneglycol dodecyl ether, Triton X-100, and 1,2-dioctanoyl-*sn*-glycerol all exhibited significant time-, concentration-, and temperature-dependent outward translocation of PS in the presence and absence of aminophospholipid flippase inhibitors. Identical results were obtained in the absence of both ATP and Ca^{2+}, eliminating any potential role of the floppase or scramblase. Pantaler et al. also examined the effects of detergents with differing alkyl chain lengths (C6-C14) on erythrocyte membrane flip-flop.[187] The ability of the detergents to induce NBD-PC flip increased with the length of the alkyl chain, as monitored by albumin back-extraction and dithionite reduction. The nonionic detergents octaethyleneglycol dodecyl ether and Triton X-100 had the greatest effect on the flip rate, requiring 10-fold lower membrane concentrations than the cationic, anionic, and zwitterionic detergents. Flip induction is attributed to membrane perturbation by the polyoxyethylene domain's interaction with the interfacial region of the glycerol backbone. Fluctuating hydrophobic pores acting as flip sites may form in the membrane. Similar reasoning explains the increased flip rates of [^{14}C]lysoPC observed in the presence of 1,2-alkane diols (C4-C8) in red blood cells.[188]

B. Reconstitution of Membrane Proteins

Several naturally occurring membrane proteins have been reconstituted into lipid vesicles and found to enhance phospholipid transbilayer movement. Membrane-spanning glycophorin, the major erythrocyte sialoglycoprotein, was first shown to affect vesicle flip-flop in the late 1970s. Over 30% of externally added [^{13}C]lysoPC accumulated within 1 hr in the inner monolayer of DOPC SUVs containing four to five copies of glycophorin.[189] Similar results were obtained using the more endogenous-like probes, [^{13}C] or [^{14}C]DOPC.[190] Studies with DOPC LUVs produced identical results, indicating that membrane curvature is not an important factor.[191] However, no transbilayer movement was detected in LUVs composed of total erythrocyte lipid. It was proposed that discontinuities were introduced into the lipid bilayer in the presence of the membrane-spanning protein.[189–191] The cylindrical shape of DOPC cannot accommodate the hydrophobic mismatch regions formed at the boundary regions, however, the wide range of erythrocyte lipid shapes (notably PE) allows for proper sealing of the bilayer. The discovery of permeable DOPC/glycophorin LUVs provided evidence for this hypothesis.[192] The 15–18 Å pores most likely result from the aggregation of several protein molecules.[193] Permeability was significantly diminished when total erythrocyte lipids were employed.[192] Early beliefs that glycophorin played a role in maintaining erythrocyte bilayer asymmetry were proven wrong when the transbilayer distribution in cells lacking the protein was shown to be normal.[194]

Flip enhancement, however, is not specific for glycophorin. Partially purified erythrocyte band 3 protein reconstituted into PC vesicles has a similar ability of inducing lipid translocation[195] and enhancing permeability which is significantly diminished in the presence of erythrocyte lipids.[196] Likewise, the incorporation of intrinsic mitochondrial membrane proteins into synthetic vesicles also affects flip rates. For example, external addition of cytochrome b_5 to asymmetric PC/PE (9:1) SUVs resulted in an initial burst of PE flop as detected by the TNBS assay.[197] A much slower translocation phase followed, indicating that addition of the protein causes a transient destabilization that is relieved through flip-flop. However, there are two literature accounts of cytochrome b_5 having no effect on phospholipid translocation. Nordlund et al. observed no change in PE

distribution upon addition of the protein to symmetric POPE/POPC vesicles.[198] Perhaps, the use of symmetric vesicles prevented the initial burst of PE movement from being detected through TNBS labeling.[197] Another laboratory reported that protein incorporation at the time of vesicle preparation using a detergent dialysis technique did not result in an increase of the exchangeability of [^{14}C]PC by the PC exchange protein.[199] Cytochrome b_5 was never added externally to the vesicle solution, however, eliminating the transient destabilization discussed in the earlier study.[197] Other intrinsic mitochondrial membrane proteins have also been investigated. Vesicles reconstituted with cytochrome *P*-450 demonstrated rapid flip-flop of [^{14}C]PC, as monitored with phospholipid exchange proteins.[199] In contrast, incorporation of cytochrome oxidase into PC vesicles had no effect on transbilayer movement.[200]

Recently, the effect of membrane-spanning synthetic peptides on phospholipid translocation was studied in a bacterial-mimicking system.[201] Using LUVs composed of *Escherichia coli* phospholipids, the half-life for outward translocation of NBD-PG was reduced to 10 min in the presence of an α-helical peptide (GKKL$(AL)_{12}$KKA), whereas movement in the absence of the peptide was insignificant. These results suggest that the high rate of endogenous phospholipid flip-flop in bacterial membranes may arise from nonspecific local perturbances created by membrane-spanning proteins. This proposed mechanism appears to be distinct from the specific translocase enzymes believed to control flip-flop in the erythrocyte membrane.

C. Electroporation

Exposure of bilayer structures to electric field pulses at 0°C induces the formation of aqueous pores which reseal upon heating to 37°C. It has been postulated that the pores form due to an ionic interfacial polarization causing water to enter the membrane.[202] The first evidence of these aqueous pores acting as flip-flop sites was offered by Dressler et al. in 1983.[203] After dielectric breakdown (5–5.5 kV/cm) and subsequent resealing, phospholipase A_2 digestion of human erythrocyte ghosts revealed that 42% of the PE and 10% of the PS had flopped to the outer monolayer. Likewise, the flip rate of exogenous [^{14}C]lysoPC drastically increased in electroporated/resealed cells; the half-life of translocation was reduced from 16 to 1.5 hr. The half-life in leaky, nonresealed cells was $<$ 15 min. In further studies, the flip rates of NBD-lipid analogs were found to decrease in the order NBD-PE $>$ NBD-PC$\gg$NBD-PS$\ggg$ NBD-SM.[204] The enhanced flip rate of NBD-PC persisted upon resealing, only diminishing by 30% in 24 hr. Upon resealing at 37°C the large defects/pores are no longer present in the bilayer, however, alteration of membrane proteins may create hydrophobic mismatch regions or nonbilayer structures.[203,204] The extent of transbilayer distribution in these cells depends upon the electric field strength and the number of pulses applied.

Cell morphology changes accompany the increased flip-flop rates produced by electroporation. For example, echinocytes form rapidly (within seconds[205]) upon electroporation at 0°C.[206] When heated to 37°C, they slowly revert to discocytes which transform further into stomatocytes. Increasing the number of electric field pulses (7 kV/cm) to 20 augments the passive phospholipid translocation process, and hardly any stomatocytosis occurs.[207] Researchers speculate that initial echinocytosis is due to a glycerophospholipid scrambling event.[206,207] Hence, the amount of lipid in the outer monolayer is effectively increased because SM remains immobile under these conditions. Only upon resealing at 37°C does SM begin to scramble, facilitating discocyte shape formation.[207] The discocytes have a symmetric lipid distribution, as determined by phospholipase A_2 and sphingomyelinase degradation.[208]

D. Pore-Forming Peptides

Similar to electrically induced pore formation, a wide range of pore-forming peptides are known to enhance phospholipid translocation. An early example that appeared in 1981 was the

voltage-controlled pore forming peptide, alamethicin.[209] Only under conditions in which the channel was open did rapid flip-flop occur. Since then, there have been numerous examples of pore-mediated flip-flop.

The antibiotic amphotericin B induces a concentration-dependent increase in the flip rate of [^{14}C]lysoPC in human erythrocytes accompanied by a redistribution of PE to the outer monolayer.[180] The antibiotic associates to form decameric water filled pores. Nearly identical results were obtained when the cytotoxin proteins from *Staphylococcus aureus* and *Pseudomonas aeruginosa* were added to red blood cells. These proteins are known to form hexameric water filled pores. The formation of such water-filled pores has also been established for the synthetic peptide, GALA.[210] This amphipathic peptide forms an α-helix when the pH is reduced from 7.4 to 5.0. The helix interacts strongly with bilayers, inducing both fusion and leakage. The membrane perturbation is accompanied by a concentration-dependent enhancement of NBD-PC flip in synthetic vesicles at a peptide/phospholipid ratio of 1:100. The authors speculate that the polar lipid head-groups reside in the water-filled pore as they travel from one monolayer to the other, while the acyl chains remain anchored in the hydrophobic domain. An additional pore-forming peptide, melittin, also facilitates phospholipid translocation, presumably by the same mechanism.[210,211]

Matsuzaki et al. have thoroughly investigated the pore-mediated flip-flop mechanism induced by the antimicrobial peptides mastoparan X[212] and magainin 2.[213] At peptide/phospholipid ratios above 1:100 and 1:200, respectively, the peptides enhance the flip of acidic NBD-phospholipids in synthetic vesicles; flip rates are independent of head-group. The researchers employed a variation of the dithionite reduction method in which the peptides were destroyed proteolytically before dithionite addition to avoid leakage of the reduction agent through the pores. In their proposed mechanism, the peptide first binds to the bilayer surface in a parallel fashion. Then, a cluster of peptides spontaneously translocates into the membrane and forms a transient water-filled pore. This pore is a supramolecular complex in which rows of lipids intercalate the helical peptides oriented perpendicular to the plane of the membrane.[214] A continuum forms between the outer and inner monolayers that serves as a passage for lipid redistribution. More recently, Hara et al. investigated the driving force for pore formation.[215] They synthesized a covalently linked anti-parallel dimer of magainin 2 which stabilized the pore and enhanced leakage. Takei et al. performed a similar analysis on melittin and revealed peptide dimerization as the rate-limiting step in pore formation.[216] Therefore, peptide oligomerization may be an essential step in pore-mediated phospholipid flip-flop.

Some antibiotic peptides induce phospholipid translocation via general membrane perturbation rather than specific pore formation. For example, the antibiotic gramicidin at a peptide/phospholipid ratio of 1:2000 induces the flip of [^{14}C]lysoPC and [^{14}C]palmitoylcarnitine in the human red blood cell.[217] Higher concentrations induce the formation of the inverted hexagonal phase. Structural defects resulting from gramicidin clusters are an essential intermediate in hexagonal phase formation and may act as flip sites. The LAGA peptide is another membrane perturbing agent that enhances flip. This peptide has the same amino acid composition as GALA, but is nonamphipathic. In synthetic vesicles, it facilitates flip-flop at a peptide/phospholipid ratio of 1:3000, but the kinetics did not fit to a standard pore model.[210]

E. Crosslinking of Membrane Proteins

Treatment of erythrocytes with thiol oxidizing agents, diamide or tetrathionate, induces extensive crosslinking of major membrane and cytoskeletal proteins through the formation of disulfide bonds. At diamide concentrations of 5 mM, the translocation rate for both [^{14}C]lysoPC and [^{14}C]PC increases dramatically in human erythrocytes.[218,219] Elevating diamide concentrations further accelerates the rate. Protein crosslinking may remove the constraints of protein scaffolding, potentially disrupting the interaction of the anionic lipids with spectrin. Another theory is that crosslinking

accelerates the formation of structural defects in the bilayer.[218,220,221] These defects may result from the protein-rich areas created, or the stretched thin portions of the bilayer containing no protein. Enhanced leakage of hydrophilic solutes accompanies crosslinking, lending support to the latter hypothesis.[220]

F. Other Means of Creating Transient Defects

There are other examples in the literature of membrane defects producing increased transbilayer movement. Raphael and Waugh generated stress differences between the monolayers of SOPC GUVs by forming thin tubes of lipid (tethers) from the vesicle surface.[222] They observed not only rapid lateral phospholipid movement, but also enhanced flip-flop. The half-life of translocation of their dinitrophenyl-PE derivative was only 8 min. Perhaps quicker lateral transport of lipids to the defect sites contributed to the significant flip-flop. Rapid lateral transport was driven by membrane stress gradients.

Bhattacharya et al. synthesized a hydrophobically modified polymer capable of catalyzing phospholipid translocation in surface-differentiated DPPC synthetic vesicles.[223] The NIPAM-C18 copolymer is a random copolymer of *N*-isopropyl acrylamide and *N*-octadecylacrylamide in approximately a 200:1 molar ratio. The compound inserts into the bilayer via the octadecyl carbon chain, and upon heating it undergoes a transition from the extended to the globular form. The transition generates a number of defects in the membrane that presumably act as flip sites. Vesicle binding via the octadecyl chain is necessary for flip enhancement because its substitution with other hydrophobic moieties such as pyrene or cholesterol did not result in the same extent of translocation.[224]

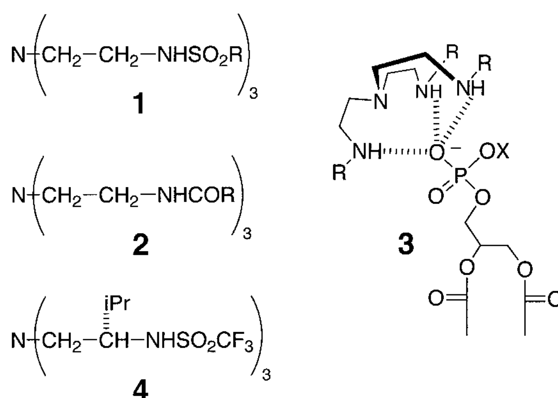
Two other studies provide additional mechanistic insight for phospholipid translocation. Homan and Pownall investigated the effect of elevated hydrostatic pressure on the flip of a pyrenyl-PE derivative in POPC vesicles.[225] Pressures up to 2 kbar decreased the rate of flip-flop, most likely due to a decrease in the number of transient defects. Another group tested the influence of altering the ionic strength of the extracellular solution on transbilayer movement of spin-labeled phospholipids in human erythrocytes.[226] Neither changes in the external surface potential or changes in the transmembrane potential had any effect on flip-flop.

7. ALTERING ASYMMETRY WITHOUT FORMATION OF TRANSIENT DEFECTS

The above examples of chemical control of transmembrane phospholipid distribution involve some type of membrane perturbation event that produces transient defects. These transient defects act as flip sites and generally allow passage of all phospholipids, regardless of head-group. Often these flip events are accompanied with membrane leakage. However, flip-flop can also be promoted by chemical agents that do not appear to disrupt the membrane.

A. Tris(aminoethyl)amine (TREN) Derivatives

Using a rational design approach, our laboratory has developed a series of low-molecular-weight synthetic translocases. Derivatives of sulfonamide **1** and amide **2** (Scheme 2) act as phosphate anion receptors in organic solvents,[227] and ^{1}H NMR binding studies reveal the sulfonamide derivatives associate more tightly with POPC than the amide derivatives.[228] The same trend is observed for phospholipid translocation of NBD-lipid derivatives across POPC vesicle membranes as monitored by the NBD/dithionite quenching assay.[229] A sulfonamide **1** derivative (R = tolyl, 38 μM) enhances the inward translocation of NBD-PC 100-fold over background with a translocation half-life of



Scheme 2. Synthetic translocases, **1**, **2**, and **4**. Structure **3** represents the proposed interaction with the PC head-group.

4 min, whereas the analogous amide **2** derivative induces only a minor enhancement. In human erythrocyte membranes, however, both the sulfonamide and amide promote the inward translocation of NBD-PC and also catalyze the reversion of echinocytes back into discocytes.[228] Control experiments verified that the TREN derivatives induce no contents leakage, no change in vesicle size, and no change in membrane fluidity. They appear to enhance flip-flop by hydrogen bonding to the phospholipid head-group, effectively reducing polarity and facilitating translocation into the hydrophobic membrane interior (Structure **3** in Scheme 2).

We have also developed synthetic translocases that selectively facilitate the translocation of NBD-PE over NBD-PC in POPC membranes.[230] The first compound is a derivative of sulfonamide **1** (R = benzo-18-crown-6, 100 μM) that reduces the half-life for translocation of NBD-PE to 100 min, presumably because of a selective interaction between the crown ether moiety and the ammonium residue on the PE head-group. Another sulfonamide derivative **4** is even more effective as a PE translocase (Scheme 2). The presence of only 8 μM translocase decreases the half-life for NBD-PE translocation to approximately 5 min, while not affecting NBD-PC movement. The effectiveness of this derivative lies in a combination of structural features. Neither of these PE translocases appear to disrupt the membrane integrity because there is no induced leakage of entrapped dyes.

Recently, the sulfonamide derivative **1** (R = tolyl) was used as a biological tool to study the peroxisome proliferator-activated receptor γ (PPARγ).[231] Upon binding specific lipid ligands, PPARγ undergoes a structural rearrangement that releases transcriptional inhibitors and recruits transcriptional co-activators. Oxidized hexadecyl azelaoyl PC (azPC) was observed to increase PPARγ activity. Furthermore, expression of PPAR-responsive genes was observed to increase in the presence of the synthetic translocase **1**, suggesting that the azPC must translocate through the cell membrane.

B. pH Gradients

Another means of altering phospholipid asymmetry without causing transient defects is to create a transbilayer pH gradient. The distribution of weakly acidic or weakly basic lipids are controlled in such a manner. In 1987, Hope and Cullis reported that stearylamine and sphingosine could be localized in the inner monolayer of DOPC LUVs with an interior pH of 5.0 and an exterior pH of 8.5.[232] They also localized oleic and stearic acid in the inner monolayer of vesicles with an internal pH of 10.0 and an external pH of 7.0. Similarly, 80–90% of the diacyl-phospholipid, egg PG, accumulates in the inner monolayer of PC vesicles with a transbilayer pH (inside basic, outside acidic) as determined by ion-exchange chromatography, ^{13}C NMR, and periodic acid oxidation.[233]

Conversely, egg PG can be trapped in the outer monolayer by reversing the pH gradient. The neutral, uncharged form of PG permeates the membrane, possibly as a dehydrated intermediate.[234] Similar PA asymmetric distributions have been generated in LUVs with transmembrane pH gradients.[233] Again, it is probably the uncharged, unprotonated form of the lipid that is transported across the bilayer.[235]

C. Other Means

High concentrations of glucose (> 5 mM) have been reported to induce changes in endogenous phospholipid distribution across the monolayers of the human erythrocyte.[236] Monitoring distribution via enzymatic degradation, a dose-dependent increase in the appearance of PS and PE in the outer monolayer, concomitant with a decrease in the appearance of SM and PC, was observed. Neither aminophospholipid flippase activity nor ATP depletion was responsible for the result. It appears that passive flip-flop is accelerated due to a secondary effect of hyperglycemia. Increases in NADPH levels associated with this state may stimulate NADPH-dependent oxidases, and the resulting byproducts of lipid peroxidation may perturb the phospholipid asymmetry.[237]

Recently, an increase in the flip rates for NBD-PS, NBD-PC, and NBD-PE was observed upon ultraviolet A (UVA) irradiation of CHO cells.[238] The administered dose of UVA had no effect on cell viability or permeability, but it may have induced lipid peroxidation through the production of reactive oxygen species in the membrane. The results showed no ATP-dependence, indicating that neither the flippase nor the floppase were involved. The authors speculate that perhaps activation of the scramblase is involved, but further studies need to be performed.

8. SUMMARY

The existence of transmembrane phospholipid asymmetry was first reported 30 years ago. Since that time, the phospholipid distributions in a variety of membranes have been elucidated, often revealing a high degree of asymmetry. However, it should be noted that there is considerable uncertainty in the data for certain membrane types such as intracellular and bacterial membranes. In many cases there is a need to redetermine the distribution values so as to lower the uncertainties. The asymmetric arrangement appears to control an array of cellular functions. Chemicals that alter phospholipid distribution are likely to have a variety of biological and pharmaceutical applications.[231]

ABBREVIATIONS

azPC azelaoyl phosphatidylcholine
BHK-21 baby hamster bovine kidney cell line
CHO Chinese hamster ovary cell line
CL cardiolipin
DLPC 1,2-dilauroyl-*sn*-glycero-3-phosphocholine
DOPC 1,2-dioleoyl-*sn*-glycero-3-phosphocholine
DPPC 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine
FMMP formyl-methionyl sulfone methyl phosphate
GUV giant unilamellar vesicle
IAI isethionyl acetimidate
LUV large unilamellar vesicle
MDBK Maden Darby bovine kidney cell line
NBD 7-nitrobenz-2-oxa-1,3-diazol-4-yl

PA	phosphatidic acid
PC	phosphatidylcholine
PE	phosphatidylethanolamine
PG	phosphatidylglycerol
PI	phosphatidylinositol
PIP	phosphatidylinositol 4-monophosphate
PIP_2	phosphatidylinositol 4,5-bisphosphate
POPC	1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine
POPE	1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine
PPARγ	peroxisome proliferator-activated receptor γ
PS	phosphatidylserine
SFV	Semliki Forest virus
SM	sphingomyelin
SOPC	1-stearoyl-2-oleoyl-*sn*-glycero-3-phosphocholine
SUV	small unilamellar vesicle
TNBS	trinitrobenzenesulfonate
TREN	tris(aminoethyl)amine
UVA	ultraviolet A
VSV	vesicular stomatitis virus

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