PART I

ASSESSING TRANSMEMBRANE MOVEMENT AND ASYMMETRY OF LIPIDS

1

METHODS FOR THE DETERMINATION OF LIPID TRANSMEMBRANE DISTRIBUTION AND MOVEMENT IN BIOLOGICAL MEMBRANES

Philippe F. Devaux
Institut de Biologie Physico-Chimique, Paris, France

Andreas Herrmann
Department of Biology, Humboldt-University Berlin, Berlin, Germany

1.1 INTRODUCTION

To access the transbilayer distribution and movement of lipids and, perhaps in particular, to convince all readers that lipids really are distributed asymmetrically between the two leaflets of eukaryotic cell plasma membranes and to explain how lipid transporters were discovered in biomembranes, it is necessary to give an overview of the main techniques that were and—in many cases—are still used. Our objective here is in fact limited to give solely an overview as well as an indication of the limits of the techniques that have been used during the last 40 years. In this book, it is not possible to detail the various aspects and weakness of each technique. Details can be found in specialized publications, but also in various chapters of this book, to which

Transmembrane Dynamics of Lipids, First Edition. Edited by Philippe F. Devaux, Andreas Herrmann.

the readers will be referred. These various techniques, because they have given not always identical but still very consistent results, have allowed some definitive conclusions to be drawn. We will respond to criticisms against the use of lipid analogs carrying a reporter moiety which "could introduce artifacts." We are well aware, and will outline below, that quantitative but rarely qualitative differences with respect to the behavior of endogenous lipids can be caused by reporter moieties covalently attached to lipids. However, even assays characterizing the transbilayer organization of endogenous lipids can cause modifications of the membrane no longer comparable to the unperturbed, original situation. Nevertheless, looking back over decades of research in this field, analogs in conjunction with assays that may affect membrane properties have provided milestones in understanding the dynamics of transbilayer distribution of lipids. For example, by using spin-labeled phospholipids, two major discoveries in this field were made. First, early in the 1970s, Kornberg and McConnell were able to give for the first time quantitative data on kinetics of passive transbilayer movement (flip-flop) of phospholipids in a bilayer [1]. Second, in 1984, Seigneuret and Devaux discovered by using short-chain spin-labeled lipids that the inward translocation of aminophospholipids in red blood cell membranes is ATP dependent, pointing to an energy-dependent lipid transporter that may also be typical for the plasma membrane of other mammalian or even all eukaryotic cells [2]. Indeed, subsequently similar conclusions of an ATP-dependent inward translocation of aminophospholipids were obtained from studies with fluorescent [3–5] as well as nonlabeled short-chain exogenous [6, 7] or even radioactive long-chain lipids [8]. Hence, being aware that labeling of lipids by reporter moieties affects their properties, lipid analogs provided significant insights into the transbilayer movement and distribution of lipids. Nevertheless, independent complementary methods, in particular those based on endogenous lipids, or at least long-chain lipids with nonperturbing labels (e.g., radioactive), are not only desirable but even mandatory to reach confident conclusions. However, applications of labeled lipid analogs are typically easier to perform as techniques relying on natural lipids. Hence, starting with lipid analogs to address questions on transbilayer lipid organization might efficiently pave the way to apply or even to develop subsequent techniques based on endogenous lipids.

1.2 DEVELOPMENT OF ASSAYS FOR DISTRIBUTION AND TRANSLOCATION OF LIPIDS ACROSS MEMBRANES

Discoveries of essential aspects of transbilayer lipid organization, in particular of plasma membranes, have been driven by the development of new methods. Likewise, questions that could not be solved with the available repertoire of methods initiated new methods. Early studies on transbilayer organization of lipids addressed whether phospholipids are asymmetrically distributed across

the membrane. Those studies investigated essentially the transbilayer distribution of endogenous lipids in the plasma membrane of red blood cells, for example, by phospholipases or by chemical modifications of lipids (see below). They revealed that aminophospholipids phosphatidylserine (PS) and phosphatidylethanolamine (PE) are preferentially localized on the cytoplasmic leaflet of red blood cells, while phosphatidylcholine (PC) and sphingomyelin (SM) are predominantly on the exoplasmic leaflet (see reviews by Op den Kamp [9, 10]). In particular, PS, contributing to about 10% of the total phospholipid content of the plasma membrane, was almost exclusively shielded from the external leaflet. These studies also implicated that the asymmetric phospholipid distribution in the plasma membrane is typical for mammalian cells. Of course, this unique distribution immediately raised the question about the molecular mechanism not only generating but also preserving the asymmetric distribution, for example, in the case of the red blood cell circulating for about 120 days in human blood vessels. So, the question was, can lipid asymmetry be created by spontaneous segregation of lipids between the two leaflets of a membrane?

Lateral segregation of lipids in domains, for example, in cholesterol- and sphingolipid-enriched domains, so-called rafts [11–14], is a thermodynamic phase separation. In contrast, transversal segregation of lipids does not exist spontaneously in a pure lipid bilayer. However, several factors may trigger such segregation. Membrane bending can cause a spontaneous segregation determined by the size of the polar head group of the lipids, which are mixed initially. In fact, a topological asymmetry exists due to membrane curvature. If one mixes lipids with a large head group and lipids with a small head group and prepares small unilamellar vesicles by sonification, a segregation of lipids is generated due to the large membrane curvature; the preference of lipids with a large head group is to occupy the external side where the curvature allows more space (see the review by Op den Kamp [10]). Other factors spontaneously generating lipid asymmetry are feasible in biological membranes with an asymmetrical environment on each side of a membrane. Transmembrane potential generally creates an electric field that polarizes each side of a membrane. Membrane proteins are not structurally symmetrically organized across the membrane. ATPases of the plasma membrane, for example, have charged residues usually on the cytoplasmic leaflet where ATP binds. Other proteins bind on the external surface or on the cytoplasmic monolayer where the cytoskeleton is attached. One could speculate that positively charged residues of those proteins may interact preferentially with lipids carrying head groups of opposite charges such as PS, or negatively charged amino acid residues with PS via the divalent calcium ion, eventually giving rise to an asymmetric transbilayer arrangement of these lipids. This is how the asymmetrical organization of lipids was initially explained with PS being trapped on the cytoplasmic leaflet of the red blood cell plasma membrane by its negatively charged head group interacting with the cytoskeleton proteins [9, 10]. However, this concept left many questions unanswered.

A new twist in understanding the generation and maintenance of lipid asymmetry in the plasma membrane of red blood cells (and other mammalian cells) was provided by the introduction of spin-labeled and fluorescent lipid analogs. When spin-labeled aminophospholipids were inserted into the exoplasmic leaflet of human red blood cells, surprisingly, they rapidly redistributed to the cytoplasmic leaflet [2, 15]. PS analogs disappeared almost completely from the external leaflet within a few minutes at 37°C, while PE reached a stationary distribution with about 80% of the analog on the inner leaflet within about 40 minutes [2]. In contrast, spin-labeled PC and SM moved only very slowly to the cytoplasmic monolayer and remained essentially on the external side. These studies provided two exciting results: a more technical one and a heuristic one. First, the stationary distribution of spin-labeled phospholipids was very similar to that of endogenous phospholipids already known, demonstrating that lipid analogs could qualitatively mimic their endogenous counterparts. Second, there is an energy-dependent transport of specific phospholipids in the plasma membrane. The directed and fast inward redistribution of aminophospholipids immediately provided an explanation for the generation and maintenance of lipid asymmetry in the plasma membrane of human red blood cells and—as implicated by later studies—typically for mammalian cells. In contrast to the model explaining lipid asymmetry by a specific interaction of lipids with the cytoskeleton, the finding of a directed transport could explain how a cell can rapidly repair or readjust any perturbation of lipid asymmetry caused by, for example, endo- and exocytotic processes. Moreover, as mentioned above, lipid asymmetry has to remain during the lifetime of the cells, which lasts sometimes more than several days. The transverse diffusion of lipids or lipid flip-flop, even slow, should lead finally to an equilibration of the lipid distribution between the two monolayers in the absence of a mechanism of lipid distribution repair, which in practice is carried out by a transporter and a flippase protein, and requires ATP as a source of energy.

These and other results [16] obtained with lipid analogs also made clear why assays based on chemical labeling or phospholipase treatment of endogenous lipids in the way they were performed could not recognize the rapid inward motion of aminophospholipids. Comparing the time required to perform the assays with the characteristic time of inward and outward motion of phospholipids, the experimental approaches would not have been able to detect the rapid inward transport of aminophospholipids.

The observation of an energy-consuming transport of lipids triggered exciting but very challenging research on the identification and characterization of lipid transporters. Although many important details still have to be unraveled, today, we know that for the rapid inward transport of PS and PE in the plasma membrane of eukaryotic cells, a P-Type ATPase is responsible (see in particular Chapters 8–10). Meanwhile, several members of the ATP-binding cassette (ABC) transporters have been identified also to mediate transport of lipids at the expense of ATP, not only phospholipids but also other lipids, for example,

sterols (see Chapters 11 and 17). It is important to mention that there are other functions of lipid transport besides generating/preserving lipid asymmetry. Lipids can be secreted to the extracellular space via transport across the membrane (see Chapters 5, 11, and 17). Another function of lipid transporter could be to generate bending of the membrane by creating a difference of the surface area between the two monolayers of the membrane (see Chapters 2, 12, and 13). Finally, essentially based on the use of spin-labeled and fluorescent analogs, the study of transbilayer motion of lipids showed that a fast, rather unspecific rapid transbilayer scrambling of phospholipids in the plasma membrane of mammalian cells can be triggered by activation of a putative scramblase (see Chapter 7). In biogenic membranes, flip-flop of lipids is typically mediated very fast by membrane proteins (flippases) mediating an energy-independent and nonspecific redistribution of lipids (see Chapter 6).

1.3 OVERVIEW ON ASSAYS FOR MEASURING DISTRIBUTION AND TRANSLOCATION OF LIPIDS ACROSS CELLULAR MEMBRANES

Several prerequisites have to be matched by an assay to generate credible results. Of course, each assay has its limits, and the choice of an assay always depends on the information in which one is interested. That is, while an assay might be useful to detect the transbilayer distribution of endogenous lipids or lipid analogs in a cellular membrane, it may not be useful to measure the kinetics of transbilayer motion of lipids. Several reports and reviews have considered in detail which criteria have to be fulfilled in order to determine the transbilayer distribution and movement of (phospho)lipids [17–22] (see also Chapters 5 and 6). Here, we will only shortly summarize the criteria: (1) The assay has to recognize the lipid of interest on the surface of the membrane in a quantitative manner. (2) The approach must distinguish between lipid species located on one leaflet and those on the opposite leaflet of the membrane. Hence, the recognizing reagent or enzyme must not have access to both sides of the membrane; that is, it must be impermeable. (3) Exchange/redistribution of lipids between both monolayers should not occur while the assay is performed. That is, the time required to assay a specific lipid quantitatively in a leaflet must be shorter in comparison to the characteristic time of lipid transbilayer movements. (4) The amount of lipid of interest should not change during the assay, for example, due to delivery of new lipids or removal of (already modified by the assay) lipids via endocytosis and exocytosis, respectively. (5) Treatment of lipids during the assay should not modify membranes leading to a perturbation and, hence, an enhanced transbilayer redistribution of lipids during assay.

In Figure 1.1, most relevant assays for assessing transbilayer movement and/or distribution of lipid analogs as well as of endogenous lipids are summarized schematically. We will now briefly describe the various assays, focusing first on

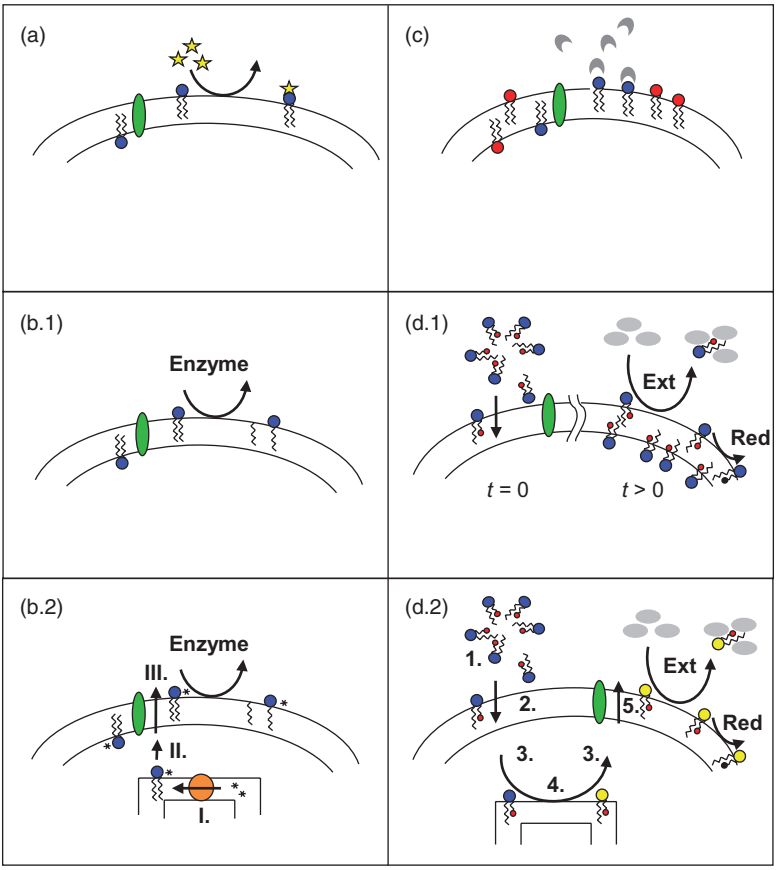

Figure 1.1. Assays for the detection of lipid transbilayer distribution (modified from Pohl et al. [81]). (a) Chemical modification assays for endogenous lipids. Endogenous lipids present on the outer plasma membrane leaflet are typically modified on the level of the head group. Reagents frequently used for modification are trinitrobenzene sulfonic acid (TNBS, specific for PE) and fluorescamine. Assays can also be applied to endogenous lipids synthesized in the presence of radioactive precursors in the cell (see b.2). (b) Enzymatic assays for endogenous lipids. (b.1) Phospholipase A2 (enzyme) treatment converts phospholipids in the outer plasma membrane leaflet to lysolipid and fatty acid. Lipid products are then analyzed by chromatography and can be compared with samples untreated with enzyme. An analogous technique is used for SM, employing sphingomyelinase. (b.2) Enzymatic assays have also been applied to assess transbilayer organization of endogenous lipids that are synthesized in the presence of radioactive precursors (marked with asterisks) in the cell (I) and localize to various cellular membranes, that is, also to the cytoplasmic leaflet of the plasma membrane, due to vesicular or monomeric transport (II), and move to the extracellular leaflet of the plasma membrane, for example, due the presence of transporter proteins (III). Upon appearance on the outer membrane surface, lipids are converted by enzyme treatment (enzyme) (see b.1).

Figure 1.1. (*Caption Continued*) (c) Antibody-, peptide-, or protein-binding assay for endogenous lipids. Specific antibodies, peptides (e.g., Ro09-198, binding to PE), or proteins (e.g., Annexin V, binding to PS) with a high affinity for a particular lipid head group bind to endogenous lipids present on the outer plasma membrane leaflet. The amount of bound antibody/peptide/protein is quantified. (d) Albumin-extraction, dithionite, and ascorbate assays for fluorescent and spin-labeled lipid analogs. (d.1) At time $t = 0$, the outer (accessible) leaflet of the membrane is labeled with short-chain spin-labeled or fluorescent analogs. Analogs redistribute to the inner leaflet of the membrane leaflet by passive flip-flop or active transport ($t > 0$). To assess transbilayer distribution, analogs are extracted from the outer leaflet (Ext), for example, phospholipids by albumin or cholesterol by methyl-β-cyclodextrin, followed by separation of cells and media. By comparing the extracted amount of analogs with that of analogs remaining in the membrane, the transbilayer distribution can be estimated. If aliquots of the sample are investigated at different time points after labeling, the transbilayer movement of the analog can be assessed. Alternatively to albumin extraction, fluorescence of lipid analogs on the outer leaflet can be quenched using dithionite, or the spin-label signal can be reduced using ascorbate (Red). (d.2) The short-chain lipid analog precursor integrates into the outer membrane leaflet (1), crosses the plasma membrane (e.g., by passive flip-flop) (2), and distributes to different intracellular membranes (e.g., by monomeric transport) (3). Enzymes of the endoplasmic reticulum or Golgi convert part of the lipid analog precursor to the lipid analog of interest (4), which can distribute back to the cytoplasmic leaflet of the plasma membrane, where it becomes available to outward transport by transporter proteins (5). Upon appearance on the outer leaflet, lipid analog is extracted (Ext) or reduced (Red) (see d.1). Color version on the Wiley web site.

endogenous lipids and subsequently on lipid analogs. Note that assays based on exogenous but nonlabeled lipids are introduced in Chapter 2.

1.4 MAIN TECHNIQUES USED TO DETERMINE TRANSBILAYER DISTRIBUTION OF ENDOGENOUS LIPIDS IN CELL MEMBRANES

1.4.1 Chemical Labeling

The first technique used to investigate the localization of phospholipids in the plasma membrane of a eukaryotic cell, the human red cell membrane, was carried out by Mark Bretscher in England in the early 1970s [23]. It was based on the chemical labeling of aminophospholipids by an NH_2 reactive reagent (Fig. 1.1a; see also Chapter 6). Typical NH_2 reactive reagents are trinitrobenzene sulfonic acid (TNBS) and fluorescamine. To match the condition that reagents do not permeate to the opposite membrane leaflet, those experiments are usually performed at lower temperatures, between 4 and 10°C. Bretscher demonstrated that PS and PE could react only if the cell membrane was made permeable to those essentially nonpermeable reagents. He was the first to claim and to demonstrate the asymmetrical organization of lipids in the human red cell membrane with the aminophospholipids PS and PE located principally

in the inner leaflet of the plasma membrane, while the choline-containing phospholipids PC and SM were principally in the outer monolayer. The bilayer concept had already been suggested a long time before by Gorter and Grendel in 1925 [24], who came to that conclusion by an evaluation of the area covered by the erythrocyte lipids. However, they could not infer the lipid asymmetry because they assumed that all lipids were identical and corresponded to phosphatidylglycerol (PG).

1.4.2 Enzymatic Treatment

Almost at the same time when Bretscher published his results with chemical labeling of aminophospholipids, the Dutch group of Laurence Van Deenen in Utrecht [25, 26] developed a completely different technique to study the transmembrane distribution of phospholipids in the plasma membrane of eukaryotes, based on lipid degradation by phospholipases and sphingomyelinases (Fig. 1.1b; see also Chapter 6). For example, phospholipase A2 treatment by addition of the enzyme to cells converts phospholipids in the outer plasma membrane leaflet to lysolipids and fatty acids. This technique seems a priori hazardous since there may be slow destruction of the membrane under investigation; analysis of the lipid composition in the time course of the assay proves that the external leaflet was indeed perturbed. Yet the results found by this assay are similar to those obtained by chemical labeling of lipids [23], namely, a preferential location of aminophospholipids in the inner leaflet of the plasma membrane. A similar distribution was also found afterward in other cells of the blood circulation such as platelets and, in fact, in all eukaryotic cells investigated with similar techniques. Small variations could be reported for different animals such as ruminants, which have essentially no PC (but a larger fraction of SM). More data corresponding to different eukaryotic cells including plant cells can be found in Chapter 3 of this book (see Table 3.1).

Another, very specific enzymatic treatment of phospholipid head group is the use of a decarboxylase, which transforms PS into PE [27, 28].

These approaches can also be applied to radioactively labeled phospholipids, allowing a more sensitive quantification of reaction products. It is very reasonable that radioactive labeling does not affect the behavior of lipids with respect to endogenous, nonlabeled lipids. Labeling of membranes with radioactively labeled phospholipids with two long fatty acid chains can be achieved by lipid transfer proteins [29–31]. However, labeling of the membrane by this technique is a rather slow process. While the approach should be useful to study the stationary distribution of lipids across the membrane, it might be difficult to resolve fast transbilayer movement of lipids.

An interesting variant is the measurement of cell surface exposure of lipids that have been labeled radioactively intracellularly. Kälin et al. incubated red blood cells with [^{14}C]-labeled fatty acids, which—after uptake—were incorporated into lyso-PC via two enzymatic steps [32] (Fig. 1.1b). Subsequently,

exposure of [^{14}C]PC on the cell surface was assessed by hydrolysis via phospholipase A2 (see above). Results were in agreement with a complementary assay based on the exchange of surface-exposed PC (including [^{14}C]PC) on PC from added liposomes by a lipid transfer protein [32].

The enzymatic approach has also been applied to lipids other than phospholipids. The transbilayer distribution of cholesterol was assessed using cholesterol oxidase [33–35] (see Chapter 5). Several enzymes and chemical modifications are available to specifically detect the transbilayer organization of glycosphingolipids (see Reference 20; Chapter 4).

1.4.3 Protein-Binding Assay for Endogenous Lipids

Peptides or proteins that bind noncovalently to phospholipids by recognizing specific parts of their head groups provide noninvasive approaches to assess exposure of endogenous lipids on the membrane surface without modification of lipids (Fig. 1.1c). In particular, fluorescent variants of these molecules allow detection of binding by fluorescence microscopy and—as very often used—by flow cytometry analysis in a rather easy way.

The most prominent example is the specific binding of Annexin V to PS, which was first employed by Thiagarajan and Tait [36]. Using Annexin V, they could demonstrate the exposure of endogenous PS on the cell surface of activated platelets (see also Chapter 7). Since then, it has found numerous applications (see Chapters 7, 15, and 16). For example, Annexin V binding is used for the detection of PS exposure as an early event of cell apoptosis [37, 38] (see Chapter 15). While binding of Annexin V to PS requires the presence of Ca^{2+}, the cell adhesion glycoprotein lactadherin recognizes PS on cell surfaces without this cation [39, 40]. Lactadherin, also known as milk fat globule-epidermal growth factor (EGF) 8 (MFG-E8), is secreted by macrophages for mediating engulfment of PS-expressing apoptotic cells. Since the activity of prothrombinase essential for blood coagulation is dependent on binding to PS on the cell surface of thrombocytes, this has been used to assay exposure of PS on the membranes [41, 42]. However, interpretation of protein binding to membranes must always consider affinity to other lipids, for example, in case of Annexin V to other negatively charged lipids.

Cinnamycin Ro09-0198 is a cyclic peptide that has been isolated from *Streptoverticillium griseoverticillatum*. This peptide specifically recognizes the head group of PE [43] and forms an equimolar complex with PE on biological membranes. To use the peptide as a probe for analyzing the surface exposure of PE, the peptide has been fluorescently labeled, preserving its reactivity and specificity [44]. For example, it has been applied to assess the exposure of PE on the cell surface of yeast cells [45]. However, one has to be aware that this peptide is able to induce transbilayer lipid movement, as has been shown for HeLa cells as well as model membranes [46]. To initiate flip-flop by Ro09-0198, the presence of PE is required.

1.5 MAIN TECHNIQUES USED TO DETERMINE TRANSBILAYER DISTRIBUTION OF LIPID ANALOGS IN CELL MEMBRANES

1.5.1 Spin-Labeled and Fluorescent Lipid Analogs

1.5.1.1 Early Studies Using Spin-Labeled Analogs The spin-labeling technique was invented by Harden McConnell, a professor of chemistry in Stanford University, in the early 1970s. (Roger Kornberg, one of his students, went on to win a Nobel Prize in 2006, on a very different subject.) In 1971 Kornberg and McConnell published a paper [1] on the slow spontaneous lipid diffusion from one leaflet to the opposite (flip-flop) in sonicated phospholipids vesicles using a modified PC (dipalmitoyl phosphatidylcholine [DPPC]), which contains in the head group a nitroxide ring (TEMPO) replacing a single CH_2. The transbilayer redistribution was measured by selective reduction of spin-labeled lipids on the outer leaflet (see below). The half-time of diffusion measured with this probe was of the order of 6 hours at 30°C [1]. Because there was no value known at the time, this number was very important to obtain. However, one could guess that TEMPO is likely to slow down the transmembrane diffusion of the lipid analog because of the size of the paramagnetic moiety and of the polar character of the probe itself. It was only in 2005 that Liu and Conboy (see below) proved by sum frequency vibrational spectroscopy (SFVS) that the TEMPO–DPPC flip-flop is indeed one order of magnitude slower compared with pure DPPC [47]. The SFVS technique is rather sophisticated but has the advantage of measuring the transmembrane diffusion of nonlabeled lipids (if one admits that deuterated lipids are indeed perfect representatives of natural lipids). The value measured by Liu and Conboy is certainly important to know, but the information obtained by the McConnell laboratory in 1971 (34 years before!) was nevertheless an extremely useful hallmark.

1.5.1.2 Spin-Labeled and Fluorescent Analogs with a Short Fatty Acid Chain The spin-labeled TEMPO–DPPC used by Kornberg and McConnell is not soluble in water because of the long chains and must be added before vesicles are formed, for example, by sonication. To label biological membranes, they have to be fused with those vesicles, or they have to be incorporated with phospholipid transfer proteins. This is a limitation that hampers the use of such probe with natural membranes even with plasma membranes like erythrocyte cell membrane.

To overcome this problem, in the early 1980s, fluorescent and spin-labeled lipid analogs, in particular, phospholipid analogs, were developed, which are slightly water soluble due to a short fatty acid chain replacing one of the natural long chains—typically in the *sn*2 position (Fig. 1.2) [2, 15, 48, 49]. The short chain usually has 5 or 6 or sometimes up to 12 carbon atoms. They actually form in water micelles and monomers, which is an essential property for efficient and rapid labeling of intact/preformed membranes. A mere addition to a suspension or monolayer of cells is sufficient to label the plasma mem-

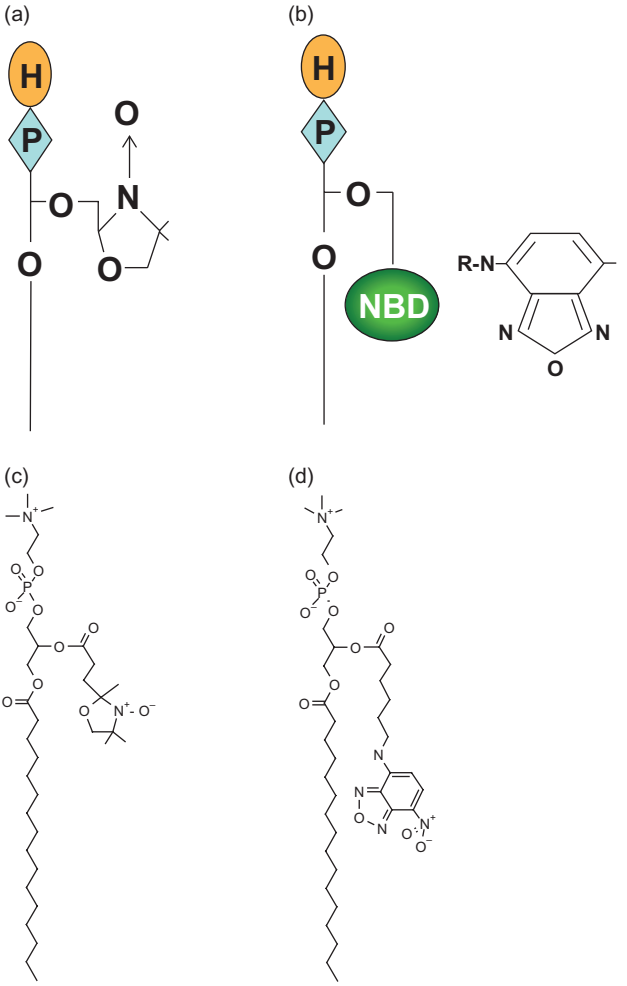

Figure 1.2. Structure of short-chain spin-labeled (a,c) and fluorescent (b,d) phospholipid analogs. Principal structure of spin-labeled (a) and fluorescent (b, NBD moiety) analogs. Chemical structures of spin-labeled (c) and NBD-labeled (d) phosphatidylcholine analogs. The label moiety is attached to the *sn2* chain. P, phosphate group; H, head group. Color version on the Wiley web site.

brane because the monomers will incorporate spontaneously. This process could be very rapid. For example, spin-labeled analogs insert within a few seconds into the outer layer of the plasma membrane [18, 19]. As a dynamic equilibrium between micelles and monomers exists, insertion of monomers into the membrane leads to a depletion and finally disappearance of micelles and monomers from the medium. Since the amount of analogs used corresponds typically to 1 mol % or even less of endogenous lipids, in many cases,

almost all analogs incorporate into the membrane. However, this has always to be checked carefully. Of course, analogs are also very useful to assess the transbilayer distribution of organelles and reconstituted systems (see, e.g., Chapters 6, 8, and 9).

The reporter moiety should be in principle as small as possible to avoid or minimize steric perturbations. Originally, most studies based on fluorescent analogs used lipids with a short chain of six carbon atoms to which terminally the fluorescent group 7-nitrobenz-2-oxa-1,3-diazol-4-yl (NBD) is attached (C6-NBD analogs). Later on, C5-BODIPY analogs were introduced, which have several advantages such as the BODIPY moiety is integrated into the lipid structure along the fatty acid chain, they are more apolar, and they have better fluorescence properties [50]. It has to be underlined that not only the fluorescent moieties but also the spin-labeled groups are of polar nature that may perturb the normal hydrophobic environment of a lipid chain. As a consequence, a looping of the fatty acid chain to which the reporter moiety (NBD) is attached (usually the short chain) to the polar interface of the membrane is facilitated [51, 52]. The possibility to attach the probe on the polar head group exists, but it raises a new problem: The analog may not be recognized by a membrane protein that is supposed to interact selectively with a lipid. In particular, a lipid has to be recognized and transported by a lipid transporter (flippase), which is usually very specific for one or a few substrates. So in practice, the reporter moieties, in particular, fluorescent groups that are generally rather big, can cause artifacts. In other words, a test of biological activity has to be done before using those probes to verify that the degree of perturbation, which is introduced by the probe, is acceptable. Alternatively, observations obtained by using lipid analogs should be verified by assessing the behavior of endogenous lipids. Indeed, there are multiple examples proving that, in particular, fluorescent probe can cause serious perturbations. Examples of artifacts due to size and polarity of fluorescent probes are given in the articles by Devaux and collaborators [22, 53].

Another important issue of those analogs is their short-chain fatty acids. Early studies have shown that the fatty acid chain may have a significant influence on lipid translocation. In 1986, Middelkoop and collaborators showed that by exposing red cell membranes to exogenous phospholipases A_2, phospholipids with at least one unsaturated chain experience a more rapid flip-flop than saturated lipids [54]. Half-times varied from 26.3 to 2.9 hours for 1,2 dipalmitoyl-PC and 1-palmitoyl-2-linenoyl-PC, respectively.

Recently, various cholesterol analogs have been studied showing large variations in the potential to mimic endogenous cholesterol [55] (for structure of spin-labeled and fluorescent sterol/cholesterol analogs, see Chapter 5).

1.5.1.3 Assessing Transbilayer Distribution and Movement of Spin-Labeled and Fluorescent Lipid Analogs In principle, there are two ways to assess the transbilayer distribution and movement of lipid analogs, either by chemical reduction of the label moieties on the outer/accessible leaflet of the membrane

by a nonpermeant agent or by extraction of analogs from the outer leaflet (Fig. 1.1d). In any case, treatment of analogs must be leaflet specific. The transbilayer distribution can be estimated from the amount of reduced/extracted analogs, that is, analogs of the outer/accessible leaflet, and the amount of nonmodified analogs, that is, analogs on the inner/nonaccessible leaflet. However, one has always to control the amount of total analogs in the membrane under investigation during the assay procedure, which in the ideal case should not alter (see below). To assess the kinetics of transbilayer distribution, aliquots of the labeled sample will be measured after different times of labeling.

1.5.1.3.1 Reduction Assay Spin labels and fluorescent NBD analogs are typically reduced by addition of ascorbate [1, 22] and dithionite [56–58], respectively (Fig. 1.1d). Both agents are, in principle, nonpermeable to membranes. Addition of the reducing agent to the suspension medium destroys the reporter moiety and, hence, the signal of labeled probes present on the outer monolayer. The remaining signal comes from probes that have flipped to the inner monolayer, which can be determined by electron paramagnetic resonance (EPR) or fluorescence spectroscopy. This allows one to determine the percentage of probes at time t that were exposed to the outer and inner monolayer. However, in case of biological membranes, and in particular at a higher temperature (37°C), reducing agents may cross the membrane reacting also with analogs on the intracellular leaflet. If so, reduction has to be performed at a low temperature, for example, 4°C. In any case, the nonpermeability of agents has to be controlled carefully (for more details, see Chapter 6). By measuring aliquots of the labeled sample at different time points after labeling the membrane, the transbilayer movement of lipid analogs can be assessed provided that the movement is significantly smaller in comparison to the time required to perform the assay, that is, reduction of analogs on the outer leaflet (for critical discussion, see Chapters 5 and 6). In principle, those assays are also applicable to analogs with two long fatty acid chains as long as the reporter moiety is accessible to reducing agent. This depends essentially on the localization of reporter moiety. If the moiety is deeply buried into the hydrophobic phase, access to them by polar reducing agents such as ascorbate or dithionite is strongly impaired. Hence, reduction may take much longer and may even be incomplete. Reporter moieties attached to short-chain fatty acids are known to loop back to the membrane surface because of its (partial) polar character (see above) becoming easily accessible to reducing agents.

1.5.1.3.2 Back-Exchange Assay Fatty acids as well as short-chain phospholipids including labeled analogs can be easily extracted from the outer/accessible membrane leaflet by albumin, typically bovine serum albumin (Fig. 1.1d), while phospholipids with two long fatty acid chains, as is typical for endogenous ones, are not removed from the membrane by albumin [16, 48, 59, 60]. Similar as described for the reduction assay, the transbilayer distribution of short-chain phospholipids analogs can be measured at a given time t after

labeling the membrane by incubation of the labeled sample, for example, of cells or organelles, with albumin for a short time (on the order of 1–2 minutes or even less [see Reference 17]). During this incubation, all analogs from the outer, albumin-accessible leaflet are extracted. Upon subsequent rapid centrifugation of the samples, the amount of analogs in the supernatant corresponding to albumin-extracted analogs and in the sediment containing the sample with the analogs on the inner, albumin nonaccessible leaflet can be measured again via EPR or fluorescence spectroscopy. If the amount of labeled lipid analogs initially incorporated into the outer leaflet (= 100% of analogs) remained constant during the whole procedure, it is sufficient to measure at different time points only one fraction, typically that in the supernatant. However, as often observed for biological samples, this is not the case. Apart from the fact that the analogs can be metabolically converted or degraded into other (lipid) molecules (see below), the reporter moiety could be destroyed as well. In particular, spin labels are rapidly reduced by intracellular redox systems as gluthathione [15, 61]. As analogs modified on the inner leaflet can also redistribute back to the outer leaflet, these lipids with a destroyed reporter moiety may occur also on the outer leaflet and will not be detected by the spectroscopical measurement. However, as shown for spin labels, those signals can be recovered by addition of appropriate reagents [16, 60].

The back-exchange assay can also be applied to assess the kinetics of the transbilayer movement. As in any case, the time resolution depends on the time to carry out the assay procedures. Usually, incubation of a sample with albumin and subsequent centrifugation with standard laboratory equipment takes at least 2 minutes; that is, any transbilayer movement on the order of 2 minutes or even faster cannot be resolved adequately. Figure 1.3 shows the transbilayer movement of spin-labeled phospholipids in red blood cells [62]. Those measurements have been performed with standard laboratory equipment providing a time resolution even sufficient to resolve the rapid inward movement of the spin-labeled PS at 37°C with a half-time of about 5 minutes. However, the inward redistribution of spin-labeled PS is much faster in the plasma membrane of sperm cells or osteoblasts with a half-time of ≤2 minutes [63, 64] (for reviews, see References 21 and 65–69). Here, the assay based on standard equipment is on its limit of time resolution.

In 1986, Tilley et al. measured the transbilayer redistribution of long-chain radiolabeled phospholipids inserted in the outer membrane leaflet of intact human erythrocytes with a nonspecific lipid transfer protein [8]. The transbilayer mobility and equilibrium distribution of the radiolabeled phospholipids were assessed by treatment of the cells with phospholipase A2. These experiments confirmed the selective ATP-dependent transport of aminophospholipids toward the inner membrane leaflet. Because probe insertion with a phospholipid exchange protein required at least 30 minutes incubation, and because cells and phospholipases also had to be incubated, no real kinetics could be drawn by this method. Nevertheless, partial kinetics data obtained with long-chain phospholipids using this technique [8] were consistent

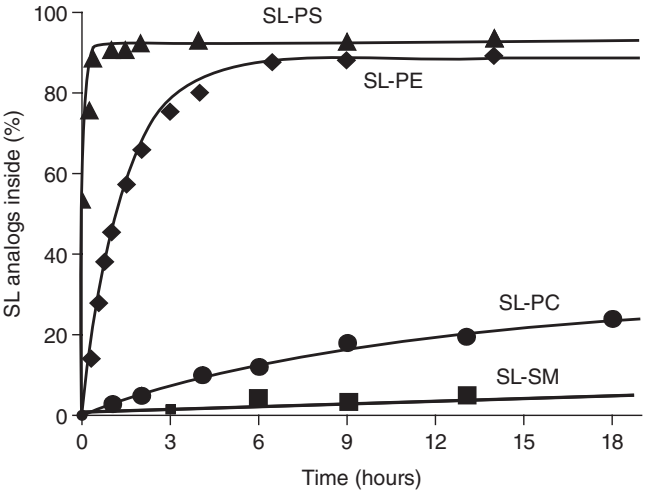

Figure 1.3. Kinetics of the redistribution of spin-labeled phospholipid analogs in human red cells at 37°C. Analogs were incorporated in the plasma membrane outer monolayer of those cells at $t = 0$ and their redistribution was followed by the back-exchange assay. The final transbilayer distributions derived from the plateaus of the curves are in fact identical to the equilibrium distribution of endogenous phospholipids in those cells (see Chapters 3 and 10). Modified from Reference [62]. Color version on the Wiley web site.

with the more detailed results obtained with spin-labeled and fluorescent phospholipids.

Buton et al. succeeded in optimizing the above-described albumin back-exchange procedure to be performed in about 30 seconds [17]. Thus, they could resolve much better the fast flip-flop of spin-labeled analogs in organelles such as the endoplasmic reticulum or the Golgi. A much better time resolution of the back-exchange assay can be obtained by employing the stopped-flow technique [18, 19] (see Chapter 6).

At this point, it might be interesting to compare spin-labeled and fluorescent analogs with respect to transbilayer distribution and movement. Again, although red blood cells are "simple cells," experiments on them nicely illustrate the differences between analogs. The short-chain fluorescent C6-NBD-PS analog redistributes much slower from the exoplasmic to the cytoplasmic leaflet in comparison with the short-chain spin-labeled PS analog [53, 62]. Furthermore, the asymmetric distribution of C6-NBD-PS is less pronounced than that observed for spin-labeled PS (see above) and endogenous PS (see Chapter 3). Hence, although still transported via the aminophospholipid translocase, the NBD moiety affects the recognition and/or transport of the analog. Interestingly, while spin-labeled PE is efficiently transported to the cytoplasmic leaflet, C6-NBD-PE is almost not transported, indicating that the fluorescent analog is a very poor substrate for the aminophospholipid translocase

[53]. Very likely, the difference between both types of analogs can be explained by the NBD group being more bulky in comparison with the spin-label moiety. Similar differences between spin-labeled and fluorescent PS analogs were observed for the plasma membrane of other mammalian cells, for example, for fibroblasts [49, 70], sperm cells [63, 71], and hepatocytes [72, 73].

Back-exchange assay has also been performed by using phospholipid vesicles as a donor of phospholipid analogs instead of albumin [74–77]. Usually, studies have been done using fluorescent analogs. Exchange of the fluorescent analog under study between acceptor and donor membranes can be followed by Förster resonance energy transfer to a stably anchored fluorescent lipid in the donor vesicles (see also Chapter 6).

While albumin is able to extract short-chain phospholipids and fatty acids, it does not extract cholesterol and sterol (analogs). However, methyl-β-cyclodextrin (MβCD) can efficiently remove cholesterol from membranes. Indeed, extraction by MβCD has been used to assess the transbilayer dynamics of cholesterol/sterol analogs. As outlined in Chapter 5, several (additional) limitations of MβCD-mediated removal of cholesterol analogs have to be taken into account. For example, those analogs in many cases barely reflect the behavior of endogenous cholesterol. A major issue is that MβCD removes not only cholesterol analogs but also endogenous cholesterol. Hence, in this case, the composition of the membrane is continuously altered. For more detailed information, see Chapter 5.

1.5.1.3.3 Consequences of Intracellular Trafficking of Analogs for Assays
Initially, phospholipid analogs have been applied successfully to characterize transbilayer distribution and movement of lipids in the plasma membrane of human red blood cells. Although one has to take into account hydrolysis of analogs during experiment, the absence of any endo- and exocytotic activity and of intracellular membranes in red blood cells was very favorable for applied assays. Indeed, a drawback in assessing transbilayer distribution of spin-labeled or fluorescence analogs in the plasma membrane of eukaryotic cells is the removal of analogs by endocytosis and by intracellular redistribution of analogs to organelle membranes [49, 64, 70, 78]. Hence, the amount of analogs in the membrane of interest may not be constant during the assay procedure, violating a criterion given above. Figure 1.4 illustrates these disadvantages. In Figure 1.4a, the endocytic uptake of fluorescent C6-NBD-PC into osteoblasts at 37°C is shown [64]. As known for PC, movement from the exo- to the cytoplasmic leaflet is slow. However, at the chosen temperature, endocytosis is a significant process, causing the disappearance of a significant amount if not most of the analog from the plasma membrane as visualized by the fluorescent intracellular spots corresponding to endosomal structures. In contrast, the fluorescent PS analog, which is transported rapidly from the exo- to the cytoplasmic leaflet by the aminophospholipid translocase activity, disappears from the cytoplasmic leaflet and, thereby, from the plasma membrane to the cytoplasm due to the partial water solubility of analogs. Finally, it inserts

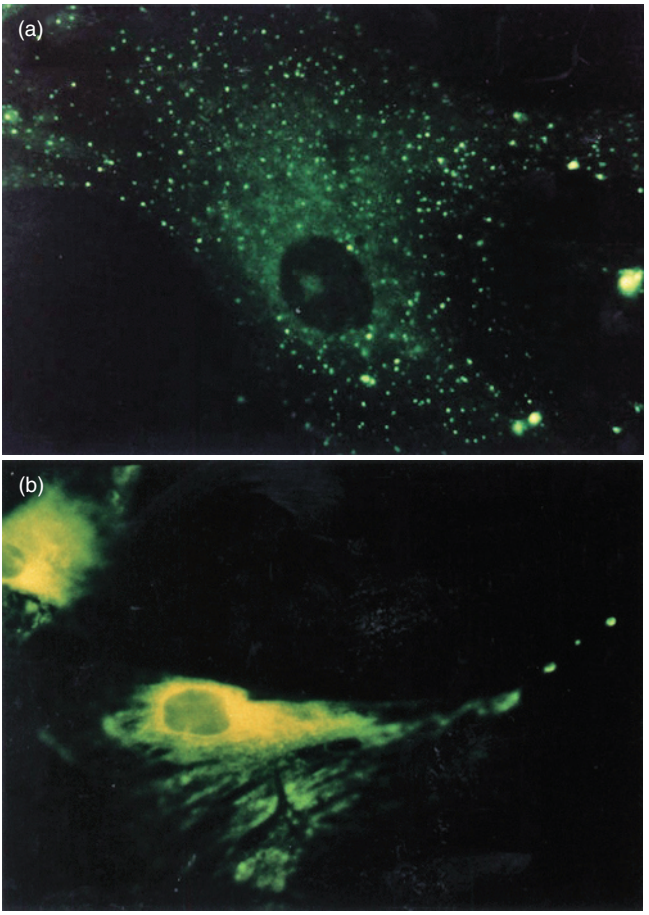

Figure 1.4. Uptake of fluorescent lipid analogs into osteoblasts. (a) Intracellular uptake of C6-NBD-PC occurs essentially via endocytosis. The yellow dots correspond to vesicles endocytosed; (b) C6-NBD-PS is transported by the aminophospholipid translocase to the ctyoplasmic leaflet of the plasma membrane. Once the probe is exposed to the cytosol, it can redistribute to other intracellular membranes because of its comparatively large solubility in water. See also Reference 64. Labeling was performed by incubation of analogs with cells. Upon incubation, noninserted analogs as well as analogs on the outer leaflet were removed by washing with albumin (see text).

into intracellular membranes, which leads to a bright intracellular staining (Fig. 1.4b). Due to the very rapid uptake of the PS analog by inward transport, the amount of PS taken up by endocytosis is low.

In addition, biochemical modifications—apart from hydrolysis—can reduce the amount of lipid analogs in the membrane during the assay procedure (see Section 1.5.2).

1.5.2 Biosynthetic Labeling

Nevertheless, the disadvantage of uptake and intracellular redistribution of (short-chain) analogs when assessing transbilayer organization of lipid analogs in the plasma membrane could be advantageous for biosynthetic labeling of lipids. The idea is that a labeled lipid analog, typically a fluorescent one, is taken up by the cell and, by metabolic processes, converted into another lipid analog (Fig. 1.1d). The trafficking of the latter, in particular, its exposure on the cell surface, is then followed, for example, by back-exchange. Thus, to examine outward transport of short-chain lipid analogs, for example, C6-NBD-PC, by the multidrug resistance ABC transporter MDR1 Pgp expressed in the plasma membrane, cells were incubated with C6-NBD-PA. This lipid analog is partially converted into C6-NBD-diacylglycerol at the plasma membrane, which rapidly crosses the plasma membrane and becomes available for intracellular synthesis of not only C6-NBD-PC but also C6-NBD-PE [79–81]. The time-dependent appearance of the fluorescent phospholipids in the extracellular membrane surface was followed by incubating the cells with albumin and measuring the fluorescence of the supernatant after centrifugation. A similar approach has been applied to measure sphingolipid translocation. Short-chain analogs of ceramide, for example, C6-NBD-ceramide, were incorporated into the extracellular leaflet of the plasma membrane. These analogs flipped rapidly across the plasma membrane and—upon intracellular redistribution from the cytoplasmic leaflet—were converted to fluorescent SM and glucosylceramide (GlcCer) by enzymes of the Golgi apparatus. Exposure of these intracellularly synthesized fluorescent lipids on the cell surface was measured by the back-exchange assay [20, 79, 82]. Those studies have shown that, for example, MDR1 Pgp is capable of transporting various short-chain GlcCer analogs to the extracellular leaflet of the plasma membrane, while the multidrug resistance transporter MRP1 was rather selectively transporting only C6-NBD-GlcCer [79, 82]. For a detailed description of transbilayer asymmetry and dynamics of glycolipids, see Chapter 4.

1.5.3 SFVS

To circumvent labeling of lipids with bulky moieties, Liu and Conboy [83] introduced SFVS for measuring transbilayer lipid movement. This coherent nonlinear optical vibrational technique, which has been described in detail [84], takes advantage of the selectivity of infrared (IR) and Raman spectroscopy. Excitation induces dipole oscillations of molecules, for example, of C–H bonds. As molecules behave like an aharmonic oscillator, overtone oscillations are excited, including the second harmonic, which is used here. Since a second-order nonlinear optical process is forbidden in media of inversion symmetry but not on surfaces with broken symmetry, this technique is surface specific in nature, which—under certain conditions (see below)—makes it also suitable for assessing transbilayer distribution and motion of lipids. Experimentally, the

excitation is read out by overlapping spatially and temporally a visible and a tunable IR laser on a surface (e.g., membrane surface). As a result, a signal (photon) is generated at the sum of their frequencies, which increases on resonance with a vibrational transition.

Conboy and coworkers generated asymmetric lipid bilayers: One of the two monolayers contained phospholipids with a deuterated terminal CH_3 group. Hence, the transition dipole moments of the C–H vibrational modes that are oriented antiparallel along the C–C bond are different between the two leaflets and do not cancel out, which can be monitored by SFVS. The difference between the transition dipole and therefore the signal becomes reduced and, finally, disappears upon transbilayer redistribution of phospholipids. Using this approach, the flip-flop of various phospholipids in bilayers of synthetic phospholipids has been measured [47, 83, 85, 86]. For example, a decrease of the fatty acid chain length of PC resulting in a significant increase in the flip-flop rate has been shown. Moreover, it was found that spin labeling can substantially affect the transbilayer dynamics of phospholipids (see above).

Although the approach requires deuterated lipids, this modification is small in comparison with tagging of lipids by spin or fluorescent labels, and the behavior of natural lipids should be preserved. However, since the technique requires the assembly of asymmetric bilayers, it is restricted to model membranes and not applicable to native, biological membranes. Nevertheless, peptides (transmembrane domains) [87, 88] and membrane proteins can be reconstituted in asymmetric model membranes to study their influence on lipid transbilayer movements.

ABBREVIATIONS

MβCD	methyl-β-cyclodextrin
NBD	7-nitrobenz-2-oxa-1,3-diazol-4-yl
PC	phosphatidylcholine
PE	phosphatidylethanolamine
PG	phosphatidylglycerol
PS	phosphatidylserine
SM	sphingomyelin

REFERENCES

1 R. D. Kornberg, H. M. McConnell, *Biochemistry* **1971**, *10*, 1111–1120.
2 M. Seigneuret, P. F. Devaux, *Proc. Natl. Acad. Sci. U.S.A.* **1984**, *81*, 3751–3755.
3 J. Connor, A. J. Schroit, *Biochemistry* **1987**, *26*, 5099–5105.

4 J. Connor, A. J. Schroit, *Biochemistry* **1988**, *27*, 848–851.

5 J. Connor, K. Gillum, A. J. Schroit, *Biochim. Biophys. Acta* **1990**, *1025*, 82–86.

6 D. L. Daleke, W. H. Huestis, *Biochemistry* **1985**, *24*, 5406–5416.

7 D. L. Daleke, W. H. Huestis, *Cell Biol.* **1989**, *108*, 1375–1385.

8 L. Tilley, S. Cribier, B. Roelofsen, J. A. F. Op den Kamp, L. L. M. van Deenen, *FEBS Lett.* **1986**, *194*, 21–27.

9 J. A. F. Op den Kamp, *New Comp. Biochem.* **1981**, *1*, 84–125.

10 J. A. F. Op den Kamp, *Annu. Rev. Biochem.* **1979**, *48*, 47–71.

11 K. Simons, E. Ikonen, *Nature* **1997**, *387*, 569–572.

12 E. Ikonen, K. Simons, *Semin. Cell Dev. Biol.* **1998**, *9*, 503–509.

13 K. Simons, E. Ikonen, *Science* **2000**, *290*, 1721–1726.

14 D. Lingwood, K. Simons, *Science* **2010**, *327*, 46–50.

15 M. Seigneuret, A. Zachowski, A. Herrmann, P. F. Devaux, *Biochemistry* **1984**, *23*, 4271–4275.

16 M. Bitbol, P. F. Devaux, *Proc. Natl. Acad. Sci. U.S.A.* **1988**, *85*, 6783–6787.

17 X. Buton, G. Morrot, P. Fellmann, M. Seigneuret, *J. Biol. Chem.* **1996**, *271*, 6651–6657.

18 U. Marx, G. Lassmann, K. Wimalasena, P. Müller, A. Herrmann, *Biophys. J.* **1997**, *73*, 1645–1654.

19 U. Marx, G. Lassmann, H.-G. Holzhütter, D. Wüstner, P. Müller, A. Hohlig, J. Kubelt, A. Herrmann, *Biophys. J.* **2000**, *78*, 2628–2640.

20 D. J. Sillence, R. J. Raggers, G. Van Meer, Assays for transmembrane movement of sphingolipids. In *Methods in Enzymology: Part B: Sphingolipid Metabolism and Cell Signaling*, Y. A. Hannun, Jr. and A. H. Merrill, eds. Academic Press, San Diego, **2000**, 562–579.

21 A. Zachowski, *Biochem. J.* **1993**, *294*, 1–14.

22 P. F. Devaux, P. Fellmann, P. Herve, *Chem. Phys. Lipids* **2002**, *116*, 115–134.

23 M. S. Bretscher, *Science* **1973**, *181*, 622–629.

24 E. Gorter, F. Grendel, *J. Exp. Med.* **1925**, *41*, 439–443.

25 A. J. Verkleij, R. F. A. Zwaal, B. Roelofsen, P. Comfurius, D. Kastelijn, L. L. M. van Deenen, *Biochim. Biophys. Acta* **1973**, *323*, 178–193.

26 R. F. A. Zwaal, B. Roelofsen, P. Comfurius, L. L. M. van Deenen, *Biochim. Biophys. Acta* **1975**, *406*, 83–96.

27 Q. X. Li, W. Dowhan, *J. Biol. Chem.* **1988**, *263*, 11516–11522.

28 A. Herrmann, M. J. Clague, A. Puri, S. J. Morris, R. Blumenthal, S. Grimaldi, *Biochemistry* **1990**, *29*, 4054–4058.

29 D. B. Zilversmit, *Methods Enzymol.* **1983**, *98*, 565–573.

30 R. L. Jackson, J. Westerman, K. W. A. Wirtz, *FEBS Lett.* **1978**, *94*, 38–42.

31 J. M. Graham, J. A. Higgins, J. A. Higgins, *Methods Mol. Biol.* **1994**, *27*, 125–130.

32 N. Kälin, J. Fernandes, S. Hrafnsdottir, G. van Meer, *J. Biol. Chem.* **2004**, *279*, 33228–33236.

33 J. Backer, E. A. Davidowicz, *J. Biol. Chem.* **1981**, *256*, 13272–13277.

34 D. L. Brasaemle, A. D. Robertson, A. D. Attie, *J. Lipid Res.* **1988**, *29*, 481–489.

35 Y. Lange, J. Dolde, T. L. Steck, *J. Biol. Chem.* **1981**, *256*, 5321–5323.

36 P. Thiagarajan, J. F. Tait, *J. Biol. Chem.* **1990**, *265*, 17420–17423.

37 S. J. Martin, C. P. M. Reutelingsberger, G. A. M. Kuijten, R. M. J. Keehnen, S. T. Pals, M. H. J. van Oers, *J. Exp. Med.* **1995**, *182*, 1545–1556.

38 F. A. Kuypers, R. A. Lewis, M. Hua, M. A. Schott, D. Discher, J. D. Ernst, B. H. Lubin, *Blood* **1996**, *87*, 1179–1187.

39 S. K. Dasgupta, P. Guchhait, P. Thiagarajan, *J. Lab. Clin. Med.* **2006**, *148*, 19–25.

40 J. Shi, Y. Shi, L. N. Waehrens, J. T. Rasmussen, C. W. Heegaard, G. E. Gilbert, *Cytometry A* **2006**, *69*, 1193–1201.

41 R. F. A. Zwaal, P. Comfurius, E. M. Bevers, *Biochim. Biophys. Acta* **1998**, *1376*, 433–453.

42 J. M. Graham, J. A. Higgins, P. Comfurius, E. Bevers, R. F. A. Zwaal, *Methods Mol. Biol.* **1994**, *27*, 131–142.

43 S.-Y. Choung, T. Kobayashi, K. Takemoto, H. Ishitsuka, K. Inoue, *Biochim. Biophys. Acta* **1988**, *940*, 180–187.

44 Y. Aoki, T. Uenaka, J. Aoki, M. Umeda, K. Inoue, *J. Biochem.* **1994**, *116*, 291–297.

45 U. Kato, K. Emoto, C. Fredriksson, H. Nakamura, A. Ohta, T. Kobayashi, K. Murakami-Murofushi, T. Kobayashi, M. Umeda, *J. Biol. Chem.* **2002**, *277*, 37855–37862.

46 A. Makino, T. Baba, K. Fujimoto, K. Iwamoto, Y. Yano, N. Terada, S. Ohno, S. B. Sato, A. Ohta, M. Umeda, K. Matsuzaki, T. Kobayashi, *J. Biol. Chem.* **2003**, *278*, 3204–3209.

47 J. Liu, J. C. Conboy, *Biophys. J.* **2005**, *89*, 2522–2532.

48 R. G. Sleight, R. E. Pagano, *J. Cell Biol.* **1984**, *99*, 742–751.

49 O. C. Martin, R. E. Pagano, *J. Biol. Chem.* **1987**, *262*, 5890–5898.

50 R. E. Pagano, O. C. Martin, H. C. Kang, R. P. Haugland, *J. Cell Biol.* **1991**, *113*, 1267–1279.

51 A. Chattopadhyay, E. London, *Biochemistry* **1987**, *26*, 39–45.

52 D. Huster, P. Müller, K. Arnold, A. Herrmann, *Biophys. J.* **2001**, *80*, 822–831.

53 M. Colleau, P. Herve, P. Fellmann, P. F. Devaux, *Chem. Phys. Lipids* **1991**, *57*, 29–37.

54 E. Middelkoop, B. H. Lubin, J. A. F. Op den Kamp, B. Roelofsen, *Biochim. Biophys. Acta* **1986**, *855*, 421–424.

55 H. A. Scheidt, P. Müller, A. Herrmann, D. Huster, *J. Biol. Chem.* **2003**, *278*, 45563–45569.

56 J. C. McIntyre, R. G. Sleight, *Biochemistry* **1991**, *30*, 11819–11827.

57 T. Pomorski, A. Herrmann, A. Zachowski, P. F. Devaux, P. Müller, *Mol. Membr. Biol.* **1994**, *11*, 39–44.

58 T. Pomorski, A. Herrmann, B. Zimmermann, A. Zachowski, P. Müller, *Chem. Phys. Lipids* **1995**, *77*, 139–146.

59 W. L. Bergmann, V. Dressler, C. W. M. Haest, B. Deuticke, *Biochim. Biophys. Acta* **1984**, *769*, 390–398.

60 J.-Y. Calvez, A. Zachowski, A. Herrmann, G. Morrot, P. F. Devaux, *Biochemistry* **1988**, *27*, 5666–5670.

61 A. Rousselet, A. Colbeau, P. M. Vignais, P. F. Devaux, *Biochim. Biophys. Acta* **1976**, *426*, 372–384.

62 G. Morrot, P. Herve, A. Zachowski, P. Fellmann, P. F. Devaux, *Biochemistry* **1989**, *28*, 3456–3462.

63 K. Müller, T. Pomorski, P. Müller, A. Zachowski, A. Herrmann, *Biochemistry* **1994**, *33*, 9968–9974.

64 J. Libera, T. Pomorski, O. JosimovicAlasevic, K. G. Fritsch, A. Herrmann, *J. Bone Miner. Res.* **1999**, *14*, 690–699.

65 P. F. Devaux, *Biochemistry* **1991**, *30*, 1164–1173.

66 A. J. Schroit, R. F. A. Zwaal, *Biochim. Biophys. Acta* **1991**, *1071*, 313–329.

67 P. F. Devaux, A. Zachowski, *Chem. Phys. Lipids* **1994**, *73*, 107–120.

68 A. Menon, *Trends Cell Biol.* **1995**, *5*, 355–360.

69 T. Pomorski, J. C. M. Holthuis, A. Herrmann, G. van Meer, *J. Cell Sci.* **2004**, *117*, 805–813.

70 T. Pomorski, P. Müller, B. Zimmermann, K. Burger, P. F. Devaux, A. Herrmann, *J. Cell Sci.* **1996**, *109*, 687–698.

71 A. Kurz, D. Viertel, A. Herrmann, K. Müller, *Reproduction* **2005**, *130*, 615–626.

72 P. Müller, T. Pomorski, S. Porwoli, R. Tauber, A. Herrmann, *Hepatology* **1996**, *24*, 1497–1503.

73 A. Tannert, D. Wüstner, J. Bechstein, P. Müller, P. F. Devaux, A. Herrmann, *J. Biol. Chem.* **2003**, *278*, 40631–40639.

74 R. E. Pagano, O. C. Martin, A. J. Schroit, D. K. Struck, *Biochemistry* **1981**, *20*, 4920–4927.

75 J. W. Nichols, R. E. Pagano, *Biochemistry.* **1981**, *20*, 2783–2789.

76 Z. M. Zhang, J. W. Nichols, *Am. J. Physiol.* **1994**, *267*, G80–G86.

77 S. Hrafnsdottir, J. W. Nichols, A. K. Menon, *Biochemistry* **1997**, *36*, 4969–4978.

78 P. Fellmann, P. Herve, T. Pomorski, P. Müller, D. Geldwerth, A. Herrmann, P. F. Devaux, *Biochemistry* **2000**, *39*, 4994–5003.

79 A. van Helvoort, A. J. Smith, H. Sprong, I. Fritzsche, A. H. Schinkel, P. Borst, G. van Meer, *Cell* **1996**, *87*, 507–517.

80 A. Pohl, H. Lage, P. Müller, T. Pomorski, A. Herrmann, *Biochem. J.* **2002**, *365*, 259–268.

81 A. Pohl, P. F. Devaux, A. Herrmann, *Biochim. Biophys. Acta* **2005**, *1733*, 29–52.

82 R. J. Raggers, A. van Helvoort, R. Evers, G. van Meer, *J. Cell Sci.* **1999**, *112*, 415–422.

83 J. Liu, J. C. Conboy, *J. Am. Chem. Soc.* **2004**, *126*, 8376–8377.

84 Y. R. Shen, *Nature* **1989**, *337*, 519–525.

85 T. C. Anglin, J. C. Conboy, *Biochemistry* **2009**, *48*, 10220–10234.

86 T. C. Anglin, M. P. Cooper, H. Li, K. Chandler, J. C. Conboy, *J. Phys. Chem. B* **2010**, *114*, 1903–1914.

87 T. C. Anglin, J. Liu, J. C. Conboy, *Biophys. J.* **2007**, *92*, L01–L03.

88 T. C. Anglin, K. L. Brown, J. C. Conboy, *J. Struct. Biol.* **2009**, *168*, 37–52.