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Transmembrane movements of lipids

A. Zachowski and P. F. Devaux

Institut de Biologie Physico-Chimique, 13 rue Pierre et Marie Curie, F-75005 Paris (France)

Summary. Membranes allow the rapid passage of uncharged lipids. Phospholipids on the other hand diffuse very slowly from one monolayer to another with a half-time of several hours. This slow spontaneous movement in a pure lipid bilayer can be selectively modulated in biological membranes by intrinsic proteins. In microsomes, and probably in bacterial membranes, non-specific phospholipid flippases allow the rapid redistribution of newly synthesized phospholipids. In eukaryotic plasma membranes, aminophospholipid translocase selectively pumps phosphatidylserine (PS) and phosphatidylethanolamine (PE) from the outer to the inner leaflet and establishes a permanent lipid asymmetry. The discovery of an aminophospholipid translocase in chromaffin granules proves that eukaryotic organelles may also contain lipid translocators.
Key words. Flippase; aminophospholipid translocase; flip-flop; lipid asymmetry.

Introductory remarks on methodologies

In 1971, Kornberg and Mc Connell showed that the transmembrane movement of spin-labeled phospholipids in sonicated vesicles is a slow process, with a half-time of several hours[67]. This was the first measurement of phospholipid flip-flop in a lipid bilayer. Shortly after, Bretscher, by chemical labeling of amino groups, discovered the asymmetric distribution of phospholipids in red blood cell membranes[17]. The two discoveries are linked: the labeling experiment undertaken by Bretscher was only possible because of the slow transmembrane diffusion of lipids. Conversely the absence of lipid exchange between the two monolayers implied de facto an asymmetrical distribution of lipids. This is because the enzymes responsible for lipid synthesis, lipid metabolism and lipid exchange are asymmetrically distributed in a living cell. Later investigations revealed a more complex situation. It appeared that the actual flip-flop rates and the percentage of inner versus outer lipid were difficult to estimate with full confidence and could vary considerably from one lipid to another or from one system to another. Premature generalization could lead to erroneous concepts. However, several independent techniques for measuring transmembrane lipid diffusion and lipid topology were developed between 1970 and 1980. These have been described in detail and critically reviewed in the often-cited articles by Op den Kamp in 1979[93] and Etemadi in 1980[38]. Here, we shall give a brief summary and point out the limitations.

Transmembrane lipid diffusion is measured by successive determinations of a transmembrane lipid asymmetry. This implies creating a lipid asymmetry with labeled lipids and having a way of monitoring a transmembrane (re)distribution. An asymmetrical distribution of spin-labeled lipids with a nitroxide probe on the polar head group can be obtained by selective chemical reduction of the nitroxide by a non-penetrating agent such as ascorbate; the subsequent transmembrane redistribution can easily be monitored by ESR spectroscopy[67]. Are the lipids which bear a paramagnetic (or fluorescent) group representative of endogenous lipids? There is actually no general answer to this question. As will be shown later in the review, spin-labeled (or fluorescent) lipids have provided invaluable data on erythrocytes and on other biological systems. Yet some of the specific lipid movements in biological membranes are inhibited when the probe is on, or near, the phospholipid head-group. In the case of real biological membranes the incorporation of labeled lipids is an obstacle which can be overcome by the use of slightly water-soluble lipids, i.e. lipids with at least one relatively short chain. Such amphiphilic lipids possess two interesting properties: 1) they can be incorporated within a minute in the outer monolayer of a cell membrane; 2) their transmembrane distribution can be monitored easily by back exchange using either sonicated liposomes or bovine serum albumin to extract the labels remaining on the outer monolayer[21,86]. However, the spontaneous diffusion of lipids depends on the exact nature of the acyl chains, as shown in erythrocytes by van Deenen's group, thus the short chain lipids may in some instances be irrelevant. Long chain phospholipids can be introduced selectively into the outer monolayer of cell membranes, if one uses a phospholipid exchange protein to catalyze the exchange between vesicles containing the probe and the biological membranes[152]. However the incorporation is slow ($\approx$ ½ h) and requires centrifugation to separate vesicles from biological membranes, and the transmembrane redistribution is not easy to monitor. The phospholipid exchange proteins can be used for this purpose as well, since the determination of the pool of labeled exchangeable lipids provides a mean of evaluating a redistribution of synthetic lipids between outer and inner monolayers. That is, providing the flip-flop rate is much slower than the exchange rate. Alternatively enzymatic degradation of membrane lipids, followed by lipid analysis on TLC, can be employed. Van Deenen's group, in Utrecht, has shown that lipid degradation by exogenous phospholipase A_2, which selectively hydrolyzes the phospholipids from the outer monolayer, is an efficient way to determine a redistribution of labeled lipids[84,129,141]. A cocktail of enzymes has to be used because of the lipid selectivity of each phospholipase. The latter technique, however, suffers from several drawbacks; it is generally slow ($\approx$ ½ h incubation), and accumulation of degradated lipids might modify the physical properties of the membrane under investigation. Thus small variations observed, for example, in pathological cells, may be insignificant.

Cholesterol transmembrane distribution has been assessed by its reaction with cholesterol oxidase, a soluble enzyme which reacts selectively with cholesterol exposed on the outer monolayer[97]. The amount of cholesterol oxidized gives, in principle, the fraction of cholesterol exposed on the outer surface. But as for the conversion of phospholipids into lyso derivatives and free fatty acids, accumulation of oxidized cholesterol modifies the physical properties of the membrane and may lead to incorrect values of cholesterol transmembrane distribution and flip-flop (see the discussion in van Meer[139]).

Yet another technique for assessing phospholipid asymmetry is chemical labeling of naturally occurring amino groups, for example with TNBS or fluorescamine (see review by Etemadi and references cited therein[38]). Nonpermeant sulfhydryl reagents can be used to localize lipids if SH groups are added as probes on suitable positions of the lipids[45]. Again, the reactions are not instantaneous and modulation of the reactivity by charged groups can interfere with any quantitative measurement of an asymmetrical phospholipid distribution. Binding of antibodies specific to particular lipids such as CL or PS could be employed in principle but, to our knowledge, no attempts to measure the diffusion of such specific lipids

have been undertaken by this technique, and it may be difficult to make it quantitative.

A new technique for measuring phospholipid flip-flop in erythrocytes was developed by Daleke and Huestis[29, 30]. The method relies on the shape changes induced in red blood cells by an increase in surface area of the inner or the outer monolayer. Phospholipids with relatively short chains can be incorporated by spontaneous exchange into the outer monolayer of erythrocytes. If this incorporation is rapid, cells become echinocytic but subsequently, if the exogenous lipids flip to the inner monolayer, they revert to the discocyte shape and eventually become stomatocytic. The time course of the shape change is a measure of the phospholipid outside-inside translocation.

From the above list of techniques it is apparent that the transmembrane distribution is generally measured by assuming a priori a slow diffusion. Since the diffusion is measured by successive determinations of lipid asymmetry only slow diffusion, i.e. with $\tau_{1/2} \gg 30$ min, can be correctly determined with most techniques. Furthermore, reliable results on asymmetry of endogenous lipids can be obtained only if the transmembrane diffusion is indeed negligible. Unfortunately, as will be shown in the following paragraphs of this review, values of half-times for lipid flip-flop cover a range extending from a few seconds for diacylglycerol or detergents to minutes for PE in bacteria or in microsomes, 10 min for PS in erythrocytes, several hours for PC in erythrocytes, days in liposomes, and months in viruses.

These introductory remarks which emphasize the difficulties in measuring lipid asymmetries and lipid flip-flop rates are meant to explain some of the contradictory or controversial data which appear in the literature.

Diffusion of uncharged lipids

This category of lipids which never bear an electric charge, is represented in cell membranes principally by cholesterol. Other neutral lipids are present only in minute amounts. They correspond often to unstable intermediates such as diacylglycerol or free fatty esters. Some of these rare lipids have very specific functions, for example sterol hormones or vitamins. Finally, many hydrophobic drugs and detergents which intercalate into membranes are neutral. The possibility that these molecules have to flip through the membranes may be an important characteristic for their physiological function. Indeed a priori these molecules, once incorporated into a membrane, will equilibrate very rapidly between both monolayers, as will molecules of organic solvents.

In plasma membranes, cholesterol corresponds to approximately 50 % of the lipids. The hydroxyl residue at position 3 of the sterol ring is probably responsible for the orientation of cholesterol within the membrane. However, this polar part does not seem to prevent fast flip-flop of cholesterol, either in artificial or in biological membranes. It must be pointed out that the characteristic times for cholesterol flip-flop reported in the literature depend on the methodology used to determine the transmembrane kinetics and vary from a few seconds to one hour[3, 5, 15, 16, 66, 71, 72]. However, half-times of the order of an hour are somewhat difficult to believe since sterol hormones or bile salts in the neutral form traverse membranes in milliseconds[20].

Diacylglycerol, which never exceeds a very low percentage of the total membrane lipids, does not exist permanently in membranes. It appears after hydrolysis of phosphatidylinositols by specific phospholipases C upon various stimuli[9] and, in turn, is quickly hydrolyzed into monoacylglycerol and fatty acid. Fast flip-flop of diacylglycerol was inferred in erythrocytes[1] and in fibroblasts[95]. More precisely the diffusion rate of sulfhydryl-labeled diacylglycerol in liposomes was characterized by a half-time of 15 s by Gannong and Bell[45]. This can be explained on the basis of the absence of charge on the molecule which creates a favorable structure for crossing the hydrophobic barrier of the interior of the membrane bilayer. Moreover, the presence of such diacylglycerols embedded in a lipid bilayer favors the occurrence of non-bilayer, hexagonal H_{II} structures[122], which destabilize the membrane and allow fast diffusion of other lipids. Thus, diacylglycerols might be considered as catalysts of transmembrane movement of lipids.

Diffusion of weakly acidic or basic lipids

These are molecules bearing a single dissociable group; at any pH, a fraction of the molecules will be charged (negatively or positively), while another fraction will be neutral. The latter fraction will behave like uncharged lipids (see above) and diffuse easily through a lipid bilayer.

Fatty acids belong to this category as their charge is derived from a carboxyl residue. Fatty acids diffuse extremely rapidly between monolayers of biological[18, 53] and artificial[34] membranes. $\tau_{1/2}$ below 1 s have been reported. In fact, the measured $\tau_{1/2}$ of diffusion is the combination of the diffusion time of the uncharged molecule and of the characteristic time for equilibrium between the uncharged and charged species. Not surprisingly, one finds very different diffusion characteristics for fatty acids according to the incubation conditions (pH, ionic strength). This property can be used to create an asymmetrical distribution of fatty acids in an artificial bilayer of phospholipids where a pH gradient has been established[58]. The fractional difference in the protonated molecules on each side of the bilayer suffices to produce an asymmetrical diffusion, and hence to allow the accumulation of the molecules on one side of the membrane. The asymmetry is immediately abolished as soon as the transmembrane pH gradient is abolished.

Weak bases such as stearylamine or sphingosine behave similarly[58], as they also exist in two states: uncharged

and protonated (here positively charged). Another category of weakly basic lipophilic molecules which have been studied in membranes are local anesthetics or tranquilizers (e.g. tetracaine or chlorpromazine). There is evidence for rapid diffusion of the free base across the membranes, whereas the protonated molecule experiences a slow penetration[37,94]. Interestingly, when chlorpromazine reaches the erythrocyte cytoplasmic surface, which is slightly acidic, it becomes charged and, subsequently, is trapped in the erythrocyte interior. The quaternary amine analogue of such anesthetics (e.g., methochlorpromazine) migrates slowly through a bilayer due to its permanent charge. Finally, the reduction of cytochrome b_5 on the inner side of the thylakoide membrane by reduced hydroquinone formed on the outer side by flash illumination takes place 10 ms after the flash[62]. By contrast semi-quinone, the corresponding charged molecule, does not diffuse through the membrane.

Among phospholipids, 4 molecules may be considered as weak acids as the only charge(s) they bear belong(s) to the phosphate group: phosphatidic acid, phosphatidylglycerol, diphosphatidylglycerol or cardiolipin, and phosphatidylinositol. In spite of the very low pK of the phosphate ($\approx 2-3$), even at physiological pH, a fraction of the molecules are uncharged and, thus, candidates for a very fast transmembrane diffusion. In liposomes, this prediction was verified for phosphatidic acid and phosphatidylglycerol; indeed, it is possible to create an asymmetric distribution of these lipids if a pH gradient is formed across the membrane bilayer[59,102]. It was demonstrated that the species which crosses the membrane is the neutral dehydrated phospholipid, and that the rate-limiting step is the dehydration of the protonated phosphate group. Cardiolipin and phosphatidylinositol exhibit a slow transmembrane motion, and no asymmetry can be generated by a transmembrane pH gradient. An explanation for this difference between PI and PG can be found in the hydration of the polar head group which bears hydroxyl groups either on the glycerol or on the inositol moieties. This shell of hydration prevents the polar group from crossing the hydrophobic core of the bilayer.

In biological membranes, such as the erythrocyte membrane, the problem may be complicated by the existence of a specific phospholipase C on the outer surface which hydrolyzes phosphatidic acid to diacylglycerol and P and thus gives rise to an artefactual, very fast translocation under physiological conditions[86,96]. If this hydrolysis is inhibited (low temperature) or prevented by using non-hydrolyzable analogs, the transmembrane diffusion of PA in plasma membrane is slow[95,119,149].

Diffusion of permanently charged lipids in artificial bilayers

This category of lipids includes zwitterionic phospholipids (SM, PC and PE) and phospholipids which bear a net negative charge on their head group, such as PS and glycolipids, as well as other lipids such as acylcarnitine, charged amphipathic drugs and charged detergents. At all pHs, these molecules contain charged residues. The passage of the polar head-group through the hydrophobic barrier limits the lipid diffusion, which very probably can only take place through defects in the bilayer. Accordingly, gangliosides, which are molecules with a very bulky head group, do not cross a synthetic bilayer even after several hours at 25 °C[118]. In general, the transfer of phospholipids between the two membrane halves is slow, with a characteristic time ranging between days and weeks depending on the bilayer composition[63,67,106,119]. This time-scale varies with the physical state of the lipids, which can be modulated by varying the temperature or the pressure[57]. The occurrence of non-bilayer structures, such as H_{II} hexagonal phases, accelerates the diffusion[89].

In some instances, integral proteins or peptides such as gramicidin may be responsible for the lipid destabilization, and hence accelerate flip-flop rates in a non-specific fashion[24]. However, results obtained in protein-containing model systems are somewhat unpredictable. For instance glycophorin, an integral protein of erythrocytes, accelerates the flip-flop rate in dioleoyl PC vesicles but is ineffective in vesicles made of lipid extracted from erythrocytes[31,136]. Two examples are favorable to the existence of short-lived 'flip' sites while proteins enter the bilayer: cytochrome b_5 causes an elevated phospholipid transverse diffusion only during its incorporation into the bilayer[48]. Once inserted into the membrane, it does not stimulate the flip-flop any more[90]. The same effect was reported for complement proteins[134]. These proteins do not seem to be 'designed' for lipid transmembrane translocation. On the other hand, it will be seen below that phospholipid flippases and translocases do exist in plasma membranes or in specific organelles. It is clear that, once inserted into artificial membranes, the latter proteins will have a permanent effect on phospholipid migration[5].

Transmembrane movement of permanently charged lipids in biological membranes

We shall consider successively three types of transmembrane movements:

a) simple diffusion; b) facilitated diffusion and c) active transport. The latter process creates an asymmetric arrangement of phospholipid between inner and outer membrane halves, while the former movements produce a random phospholipid distribution unless a driving force such as an electric field or a pH gradient segregates a fraction of the lipid population[59,60,102].

a) *Simple diffusion.* The diffusion of various species of PC has been extensively studied in biological membranes, in particular in erythrocytes. ESR[86,110], fluorescence[26], selective hydrolysis by exogenous phospholipas-

es[129,141], use of phospholipid exchange proteins[14,28], back exchange technique[44], and analysis of cell shape[29,30] are techniques which have been used successfully to measure PC transmembrane diffusion in human erythrocytes. All laboratories have reported half-times of several hours (2–10 h). The exact reorientation velocity depends on the nature of the acyl chains (length, degree of unsaturation[30,44,84,110]) and possibly on the presence of perturbing probes.

Slow motion of PC molecules was also found in plasma membranes of other types of cells: platelets[125], lymphocytes[126,151], cultured fibroblasts[112,123], and renal brush border membranes[142]. Two exceptions would be membranes from electric organ[80] and rabbit intestine brush border[6], where PC half-times of a few minutes were reported. However one should keep in mind the possibility of experimental pitfalls such as membranes being leaky to ascorbate or degraded by phospholipases; it is noteworthy that in rabbit kidney brush border, Venien and Le Grimellec[143] showed that the apparent flip-flop of PC depends on the type of phospholipase used. Thus, the results of Barsukov et al. for intestine brush border[6] should be taken with caution. Transmembrane movement of phospholipids is slow in viruses as well. A half-time in excess of 30 days was estimated in 1976 by Rothman and collaborators for influenza virus[108]. More recently, Allan and Quinn reported a significant flip-flop in 20 h in the case of Semliki Forest virus[2]. In isolated organelles from eukaryotes, slow flip-flop has also been reported[109,150]; liver microsomal membranes constitute a well-documented exception where the fast diffusion[61,133,157] is attributed to the existence of a phospholipid 'flippase' (see below). Fast transverse diffusion of PE has been reported also in bacteria[74]. In fact intrinsic proteins might be involved unspecifically in this process by perturbing the bilayer and creating local defects, which facilitate phospholipid flip-flop. We have indicated half-times for flip-flop of a few hours in erythrocytes; this is notably shorter than most values obtained for pure liposomes, where values of days have been reported. Experiments carried out in our laboratory with a series of spin-labeled phospholipids containing different head groups led systematically to the demonstration of a faster diffusion in erythrocyte ghosts than in liposomes from the lipid extract[149]. This residual diffusion shows little selectivity; for example, the charged PS molecule flips in the absence of ATP at the same rate as the zwitterionic PC molecule; SM movement on the other hand is slower. Thus, the residual transverse diffusion of phospholipids in biological membranes may be caused by bilayer destabilization at protein boundaries.

b) *Facilitated diffusion.* When specific transport proteins are involved in transmembrane movements of lipids, the rate of translocation must be saturable. Using a short chain phospholipid (dibutyryl PC), Bishop and Bell discovered a lipid translocation which was saturable and sensitive to proteases and chemical protein modifications

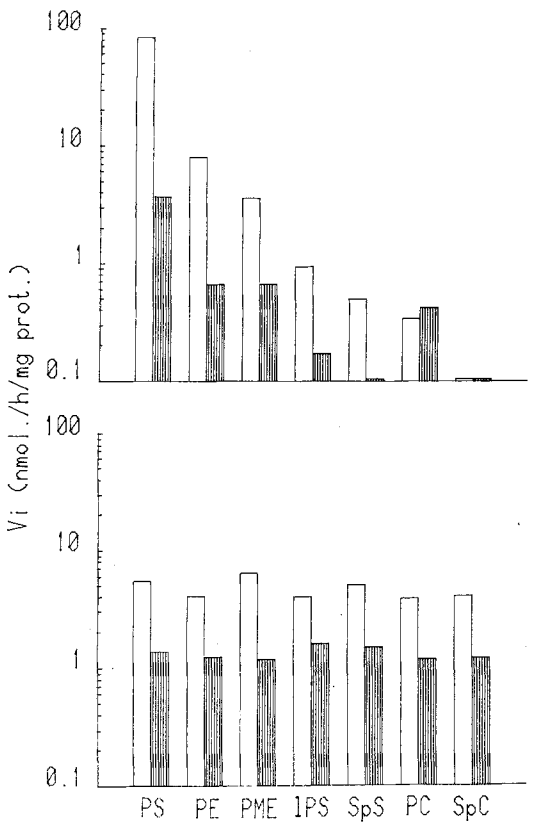

Figure 1. Comparison of the initial velocities of outside-inside translocation of spin-labeled phospholipids, at 37 °C. Above: human red blood cells; below: rat liver microsomes. The dashed bars correspond to experiments carried out after incubation with 1 mM N-ethyl-maleimide. PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; PME, monomethyl-phosphatidylethanolamine; LPS, lyso-phosphatidylserine; SpS, sphingosyl-phosphorylserine; SpC, sphingomyelin. (Adapted from Herrmann et al.[53] and Morrot et al.[86]).

in endoplasmic reticulum vesicles from rat liver[11]. Similarly, a fast, saturable equilibration of lyso PC[65] and of several other water-soluble phospholipids[53] was described in these membranes. When more hydrophobic phospholipids were incorporated into microsomes, a random distribution of the molecules between the two monolayers was observed[53]. The process was slowed down by the action of N-ethylmaleimide (see fig. 1). Bishop and Bell postulated the existence of a specific protein which they called flippase. The existence of the flippase was confirmed by reconstitution experiments using microsomal proteins: the rapid flip-flop was found only if lipids were mixed with microsomal proteins and not if erythrocyte proteins or microsomal lipids alone were used for the reconstitution[5,53,133]. The mechanism of action of this non-selective flippase is not known. Some arguments exist in favor of protein-induced formation of non-bilayer structures in microsomes. Such non-lamellar structures were revealed by ^{31}P-NMR in liver microsomal membranes and were not found in aqueous dispersions of the extracted lipids[138]. The role of this fast migration which equilibrates lipids between the two monolayers is understandable in a membrane specialized

for lipid synthesis with asymmetrically distributed enzymes[25,55,61,132]; the fast transverse diffusion avoids accumulation of products in one monolayer and allows an intermediate synthesized on one leaflet to reach the next site of synthesis on the other leaflet. In bacteria, lipids are synthesized in the cytoplasmic membrane in an asymmetrical fashion. Thus, there is a need for a flippase to allow lipid equilibration. Langley and Kennedy have reported a fast transverse diffusion of PE in *Bacillus megaterium*[74]. This mechanism is somewhat enhanced in energy-poisoned cells indicating that lipid flip-flop in bacteria is not coupled to lipid synthesis. In erythrocytes the methylation of PE to PC by the methyltransferase is accompanied by a translocation of the lipid[56]. In the mitochondrial inner membrane the carnitine-acyltransferase imports acylcarnitine into the matrix space where it is deacylated. This exchange is not affected by the energy state of the mitochondria[88].

c) *Active transport.* Active lipid translocation means that a lipid molecule is moved from a layer where it is less concentrated to a layer where it predominates. Energy, namely ATP, is consumed to overcome the chemical gradient and, in some instances, the electrical gradient. Active transport of phospholipids was first described in 1984 in human erythrocytes by Seigneuret and Devaux[115]. In this system, the outside-inside migration of spin-labeled analogs is slow for PC, but fast for PE and even faster for PS with a half-time of 5 min at 37 °C (see fig. 2). The rapid translocation of aminophospholipids does not take place in ATP-depleted cells or in ghosts resealed in the absence of ATP. The process is inhibited by vanadate, a known ATPase inhibitor; the non-hydrolyzable ATP, 5'-adenylylimidodiphosphate, cannot substitute for ATP. The rapid, ATP-dependent translocation of aminophospholipids, which was demonstrated originally by ESR, was confirmed by other techniques using long-chain radioactive phospholipids[105,129] and fluorescent phospholipids[26], and by monitoring shape changes induced by exogenous lipids[29,30]. To explain these results, we have postulated the existence of a specific enzyme, the aminophospholipid translocase, the properties of which will be summarized in the following paragraphs[13,33,86,115,148].

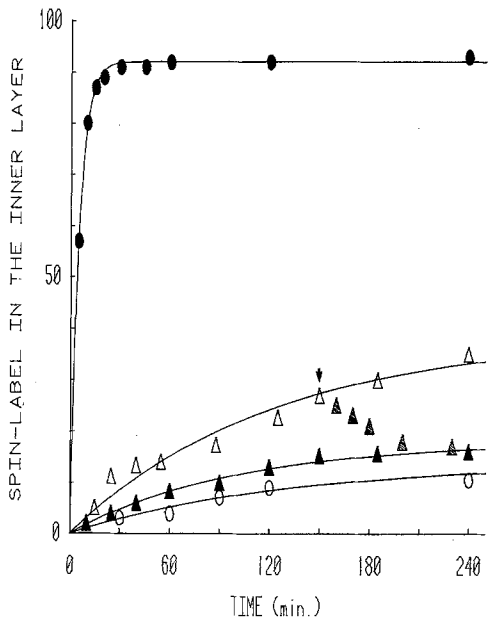

Figure 2. Kinetics of transmembrane reorientation of spin-labeled phosphatidylserine initially incorporated in the outer leaflet of: ●, erythrocytes; ○, erythrocytes depleted of ATP; △, chromaffin granules in EGTA containing buffer; at the time indicated by the arrow, Mg-ATP is added externally at a concentration exceeding that of EGTA and PS translocation is followed; ▲, granules in Mg-ATP containing buffer. (Adapted from Morrot et al. [86] and Zachowski et al.[150]).

The energy substrate is the Mg-ATP complex and its apparent K_m is in the millimolar range. The binding of Mg-ATP to its site facing the cytoplasm, and the binding of the aminolipid in the outer monolayer, are independent of each other. PS and PE are recognized and transported by the same protein, but PS is the better substrate as indicated by a ten-times lower apparent K_m. Phospholipid recognition by the translocase depends on different parts of the ligand molecule. The binding requires a dissociable amino group on the polar head; this group must be at a distance of two methylenes from the phosphate group, as phosphatidylpropanolamine has no affinity to the aminophospholipid translocase. The carboxyl residue increases the affinity of the protein for the aminolipid as indicated by the following observations: a) phosphatidylserine carboxymethyl ester is transported like PE; b) if the amino group of serine is replaced by a methyl group (serine → β-lactate), the lipid is no longer transported even though the carboxyl group is present (P. Fellmann, personal communication). If a ceramide backbone is substituted for the glycerol backbone of PS, transport efficiency decreases by almost three orders of magnitude. Finally c), the nature of the chain in sn-2 plays a role; a lyso-PS is not efficiently relocalized on the cytoplasmic face[8,86], but the presence of an acetyl group esterified on the glycerol is sufficient to restore the affinity. Daleke and Huestis have shown that saturated and unsaturated PS are transported at the same rate[86,129]. It might appear that the nature of the chain at sn-2 is unimportant, as the spin probes we used, which bear a 4-(doxyl)-pentanoyl fatty acid, exhibit similar kinetics to long-chain lipids[129]. However, this assumption has to be carefully considered, as fluorescent phospholipid analogs, whose acyl residues in sn-2 are (6-NBD) caproyl groups, are less efficiently transported, which suggests a lower affinity (M. Colleau, personal communication).

The translocase in red blood cells is not only responsible for outside-inside passage of aminophospholipids, but also controls the inside-outside movement[13]. Indeed, we have shown that: a) the outward motion of aminophospholipids is faster than that of PC, b) Mg^{2+} depletion and/or NEM treatment reduces this outward motion. Thus, the steady-state equilibrium distribution of aminophospholipids is determined by the balance be-

tween inward and outward movement through the translocase. If the translocase is blocked, the redistribution follows different kinetics, associated with the spontaneous flip-flop, and is characterized by a very long half-time.

To date, the aminophospholipid translocase has not been purified. Radioactive photoactivable PS has been used by two laboratories in an attempt to determine the molecular weight of the protein. Connor and Schroit concluded from their experiments that the translocase is a 32 kDa protein which is also labeled by sulfydryl reagents[27,114]. On the other hand, we have proposed that the aminophospholipid translocase is a vanadate-sensitive Mg^{2+}-ATPase of molecular weight 115–130 kDa, which is present in red blood cells[36] and in most plasma membranes, and whose function is as yet unknown[41]. Positive identification of the aminophospholipid translocase awaits successful reconstitution experiments.

The same active translocation of aminophospholipids, sensitive to chemical protein reagents, has been found in plasma membranes from lymphocytes[126,151], platelets[10,125], cultured hamster fibroblasts[79], and synaptosomes from the *Torpedo* electric organ (A. Zachowski and Y. Morot-Gaudry, unpublished observations). Recently, we have shown that this activity is not confined to the cell surface and can be found associated with the membrane of a cell organelle, namely adrenal medulla chromaffin granule[150]. Figure 2 shows that the activity of the ATP-dependent pump in the granules tends to accumulate PS on the external monolayer which is the cytosolic face of this organelle. Interestingly, chromaffin granules possess a membrane-bound Mg-ATPase with a molecular weight of 115 kDa, i.e. of the same molecular weight as the erythrocyte Mg-ATPase which we think is the aminophospholipid translocase.

Phospholipid asymmetry in biological membranes

Phospholipid asymmetry is well established in several plasma membranes. In human erythrocytes, the inner monolayer contains 96 ± 4 % of the PS molecules, 80 ± 5 % of the PE, 30 ± 7 % of the PC and ≈ 10 % of the SM[8,17,47,64,140,144,146]. Similar distributions with a majority of aminophospholipids exposed on the inner monolayer were found in erythrocytes from animals[28,46,49,100,103,135] and in the plasma membranes of platelets[22,78,111,113], fibroblasts[40,112,117] and ascites cells[101]; in brush borders from intestine and kidney[6,98,143], and synaptosomes from brain[39]. From experiments on lipid asymmetry in viruses, the asymmetry of the plasma membrane of the infected cells can be deduced. Such observations allow one to infer that BHK-21 and MDBK cells have a plasma membrane asymmetry comparable to that of red blood cells[2,108,142]. The only reported exception is chick myoblast cells, where PS is randomly distributed and PE is predominantly on the outer monolayer[117].

The asymmetric distribution of phospholipids is accompanied by an asymmetrical distribution of fatty acid chains. In human erythrocytes, the double bond index is 1.54 for the inner face and 0.78 for the outer face[82], principally because 66 % of SM has d 18 : 1 acyl chain in position 1, and 16 : 1, 24 : 0 or 24 : 1 acyl chains in position 2 and 80 % of PS has 18 : 0 in position 1 and 20 : 3, 20 : 4, 22 : 4, 22 : 5 or 22 : 6 in position 2. One of the consequences of this asymmetrical composition is the asymmetrical physical properties of the erythrocyte membrane. Indeed, the outer monolayer is more packed and rigid than the inner one[137,146]; the rotational mobility of spin-probes is higher in the inner monolayer[86,116,127], and the lateral diffusibility of the phospholipids is higher in the cytoplasmic leaflet[87,104]. Scrambling the phospholipid asymmetry abolishes this asymmetrical 'fluidity' and modifies properties such as lipid packing[145]. Similar asymmetric fluidity was found in fibroblasts, and probably also occurs in platelet plasma membranes, as the outer leaflet is poor in lipid unsaturation[99] and is tightly packed[22,76].

The transbilayer distribution of phospholipids in organelles is less well documented than in plasma membranes, probably because the isolation of such membranes requires cell lysis, purification and resealing, each step being the source of possible errors. Also, the existence of a fast transverse diffusion may impair the results of asymmetry determination. In many cases, such as endoplasmic reticulum, nuclear membranes and inner mitochondrial membranes, contradictory data are presented in the literature. There is agreement, however, in the case of muscle sarcoplasmic reticulum, where PC is symmetrical, PE is preferentially (60–70 %) outside and PS is preferentially (80 %) inside[51,54,131]. There are also indications that in the outer mitochondrial membrane PC and PI are randomly distributed while the greater part of the PE is inside[124]. Finally, in chromaffin granules and in gastric and synaptic vesicles, PE appears to be exposed on the outer leaflet which is the cytoplasmic leaflet[19,81,92]. This preferential exposure of PE may be necessary for the vesicles to become fusion competent. Indeed, there is a large amount of evidence suggesting that close contact between membranes is facilitated when the lipids vis-à-vis to one another are PS-PE mixtures and not PC-SM mixtures[32].

The aminophospholipid translocase, by accumulating aminophospholipids on the cytoplasmic side of eukaryotic membranes, is undoubtedly responsible for the stable asymmetric distribution of aminophospholipids in the plasma membrane. Asymmetric distribution of PC and SM may be explained as simply being the consequence of the aminophospholipids occupying the 'lipid sites' of the inner monolayer. The establishment of lipid asymmetry implies therefore some diffusion of the choline-containing phospholipids. Yet, in the case of SM,

the outside-inside movement is extremely slow, almost null[86], perhaps because of SM-SM interactions on the outer monolayer. It would be important to measure the inside-outside motion of SM which may turn out to be faster than the inward motion. In summary, we think that the lipid asymmetry of the four main erythrocyte phospholipids can be accounted for by asymmetrical lipid flows. In other words, the asymmetry corresponds to a steady state and not a static lipid distribution.

At this point it should be mentioned that some laboratories believe that cytoskeleton proteins are crucial for the maintenance of lipid asymmetry. This theory is based on the following experiments: 1) Experiments with model systems showed an interaction between spectrin and PS[85]; 2) reduced lipid asymmetry has been observed in cells wherein the cytoskeleton had been modified chemically or partially disconnected from the membrane, as is believed to happen in deoxy-sickle cells[23, 75, 83]; 3) lipid asymmetry is only scrambled slowly when cells are deprived of ATP[83, 129].

Our comments are as follows: a) biophysical experiments prove that the interaction between PS and spectrin is in fact very weak (of the order of the thermal energy[77]); b) red blood cell vesicles depleted of spectrin by thermal denaturation can still maintain and create asymmetry providing the cytosol contains ATP[21, 35]; c) experiments that modify not only the cytoskeleton but also several other characteristics of the cell such as ATP level, oxidation state of SH groups etc. cannot be conclusive. Thus it is our feeling that the role of the cytoskeleton, at least in red blood cells, has not been proven. It certainly is not implicated in organelles such as granules which have no cytoskeleton.

Hubbell has proposed to explain lipid asymmetry in disc membranes by the asymmetrical charge distribution on rhodopsin which constitutes approximately 80 % of the disc proteins. Hubbell's model includes no kinetic considerations[60]. Although the charge distribution may influence the stability of PS asymmetric distribution, Hope and Cullis have shown that an electrical gradient is not sufficient to generate an asymmetric distribution of charged phospholipids[58]. A 'flippase' would be necessary to allow lipid equilibration between both layers. Alternatively the lipid asymmetry may be created in the plasma membrane, where the discs originate, by an aminophospholipid translocase, and subsequently preserved in the discs because of the electrical gradient. In conclusion, Hubbell's model needs further experimentation.

Finally there are indications of lipid asymmetries in bacterial membranes[12, 68, 107]. A priori one would think that the asymmetry of lipids in prokaryotes is correlated with the asymmetrical synthesis of phospholipids which takes place in the bacterial membranes. However, fast phospholipid translocation was measured in the membrane of *Bacillus megaterium* (see above). Thus, the inner and outer halves of the membrane should rapidly equalize. According to Langley and Kennedy[74], this does not occur. Cells in which the generation of metabolic energy had been completely blocked by suitable inhibitors had, in fact, a somewhat increased asymmetry[74]. Perhaps an asymmetric charge distribution on the proteins could be invoked as an explanation as suggested by Hubbell for the discs. In this case PG, which is a charged lipid, might be sequestered in the inner monolayer, and PE would diffuse to the outer monolayer via a flippase comparable to that existing in microsomes.

Physiological and pathological modifications of lipid diffusion in eukaryotes

Under particular conditions corresponding for example to stimulation by activators or by drugs, cell oxidation or aging, the flip-flop rates are modified either temporarily or permanently. Also pathological cells, in particular red blood cells, often exhibit abnormal flip-flop rates. These modifications are often, but not always accompanied by a change in lipid asymmetry. The cell response to a new lipid distribution can give a clue as to the physiological role of lipid asymmetry and the importance of controlling lipid exchange between monolayers. In principle a strategy to study the translocase or the flippase would be to search for mutants deprived of the capacity to flip phospholipids. However, to date there is no demonstration of the existence of such mutants in bacteria or in yeast which could perhaps constitute systems for genetic investigations.

Perhaps the most spectacular physiological modification of lipid asymmetry is that of the plasma membrane of activated platelets. Zwaal and collaborators have shown that platelet activation is accompanied by a very rapid loss of membrane asymmetry indicating a temporary increase in transbilayer movement of phospholipids. This lipid scrambling may be due to the concomitant fusion of granules with the plasma membrane[154]. This process, which leads to increased exposure of negatively charged phospholipids at the outer surface, could play an important role in local blood-clotting reactions. Prothrombin is hydrolyzed to give thrombin, and this leads to the formation of fibrin strands and of the clot. Remarkably, if activated platelets are treated with reducing agents, PS progressively disappears from the outer leaflet, suggesting that the aminophospholipid translocase eventually corrects the sudden loss of lipid asymmetry[10].

More subtle changes in phospholipid translocation are observed in aged red blood cells and in sickle cells. Aged red blood cells can be isolated by centrifugation on a Percoll gradient. Using spin-labels, we have observed that the aminophospholipid translocation rate in the more dense (older) cells is reduced by approximately 20 % as compared to the lighter (younger) fraction[52]. This partial inhibition appears to be sufficient to reduce the final asymmetric distribution of exogenous PE, but not that of PS. Alteration of the asymmetry of endoge-

nous phospholipids in 'old' red blood cells, in accordance with the spin-label data, has been reported by Shukla and Hanahan[121]. The spin-label data show that this alteration is due to the impairment of the aminophospholipid translocase. Further experiments with malonyldialdehyde (MDA) or H_2O_2-treated cells suggest that oxidation of the protein is responsible for this impairment. The fact that PS transmembrane orientation is not significantly altered in the more dense cells may in fact mean that such a perturbation would be lethal for the cells. Indeed, Tanaka and Schroit have shown that red blood cells with PS on the outer monolayer are taken up rapidly by macrophages[128]. Thus, probably only a very small fraction of the older cells have PS on the exofacial side.

Sickle cells have similar characteristics. It has been reported that sickle cell disease is accompanied by a reduced phospholipid asymmetry[23, 75, 83], and also by a reduced aminophospholipid translocase activity[83, 147]. A reduced asymmetry was also found in Rh_{null} human erythrocytes[69]. It can be noted that in these pathological cells the passive transmembrane diffusion of PC is accelerated[42, 69], but this change cannot explain the modification of asymmetry, as other cells express a high rate of translocation of PC while keeping a normal transmembrane distribution of phospholipids[43, 70]. Finally, in malaria-infected erythrocytes an increased flip rate of the four main phospholipids present in the cell was reported by Beaumelle and collaborators[7], which is, however, not accompanied by a change of lipid asymmetry. The considerable increase of PC flip-flop led Haldar et al. to postulate the existence of a PC flippase in the membranes of parasitized red cells[50].

Conclusions and speculations

Figure 3 summarizes the various processes which control the distribution of phospholipids between the inner and outer halves of biomembranes. An implicit assumption, which we have not discussed yet, is the fast lateral diffusion of lipids in each monolayer. In fact, from the values of lateral diffusion measured, this should not be a rate-limiting step. However, the concept of lateral domains in biomembranes has recently been emphasized by Tocanne et al.[130]. Taking this concept into account, it is conceivable that not all lipids have free access to the translocation sites of the specialized proteins, which could raise additional complications.

Because of the asymmetry of some of the translocation mechanisms, most biological membranes have an asymmetrical distribution of phospholipids, the physiological roles of which may be multiple. We have discussed in the preceding section the implications of PS appearance on the exofacial surface of circulating cells; loss of PS asymmetry triggers cell-cell interactions which in some instances could lead to the elimination of the cells from the blood stream. We also pointed out that the cytosolic exposure of aminophospholipids on plasma membranes and simultaneously on organelles, such as presynaptic vesicles and granules in chromaffin cells or in platelets, favor their close contact and hence may be necessary for fusion of these organelles to the plasma membrane. Possibly phospholipid redistribution might modulate the activity of PS-dependent enzymes such as kinases and annexins. Yet another function for the control of phospholipid transmembrane distribution is connected with cell morphology. The accumulation of lipids on one leaflet of the membrane by a lipid pump tends to modify the ratio of inner and outer surfaces, and thus to modify the bending of the membrane. In red blood cells spectacular shape changes result from exogenous or endogenous lipid redistribution[31, 32, 132]. This can be explained in the framework of the bilayer couple hypothesis of Sheetz and Singer[120]. Local shape changes may take place also in nucleated cells, for example in fibroblasts, under the influence of the plasma membrane aminophospholipid translocase. Unbalanced inward transport of phospholipids should lead to invaginations of the type observed during the process of endocytosis. Thus the aminophospholipid translocase may be a key enzyme in lipid traffic within nucleated cells.

Finally, Norris has put forward an interesting theory to explain cell division in *E. coli*[91]. He postulates that a class of lipids escapes from the rapid flip-flop mecha-

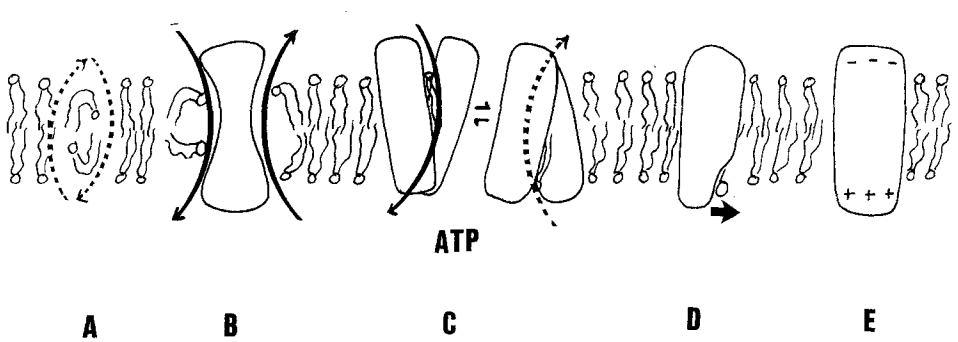

Figure 3. Schematic representation of the various mechanisms allowing phospholipid reorientation in a biological membrane. *A* spontaneous flip-flop; *B* facilitated diffusion by a phospholipid flippase; *C* active transport by the aminophospholipid translocase; *D* asymmetric lipid synthesis; *E* asymmetric charge distribution on proteins which may stabilize lipid asymmetry.

nism. These phospholipids would accumulate in the inner monolayer of the cytoplasmic membrane and thus cause an increase of its packing density; at a critical density, phospholipids would 'flip out' and initiate cycle events.

Abbreviations. PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; SM, sphingomyelin; CL, cardiolipin; NEM, N-ethyl-maleimide; TLC, thin layer chromatography.

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Concluding remarks and perspectives

G. Daum

Institut für Biochemie und Lebensmittelchemie, Technische Universität Graz, Schlögelgasse 9/III, A-8010 Graz (Austria)

The existence of subcellular compartments provides a spatial separation of metabolic events and cellular processes, which allows the coexistence and coordination of different pathways within the cell. Biological membranes form the barrier between organelles and their environment. As well as proteins, lipids are integral parts of all cellular membranes. They act as structural elements, but by providing the proper environment they also affect the activity of enzymes present in membranes by modulation. The elucidation of processes involved in the assembly of lipids into cellular membranes is therefore an important subject of modern cell biology.

The present Multi-author Review summarizes our knowledge of the supply of lipids to membranes, of mechanisms involved in intracellular lipid translocation, and of regulatory effects on the migration of lipids within the cell. Morton and Vance and Vance describe the interaction between extracellular (lipoproteins) and cellular lipids. A plasma lipid transfer protein catalyzes the migration of cholesterol esters and triacylglycerols between lipoproteins and cells, which leads to the modulation of cellular lipid levels and directly influences cellular membrane lipid composition. Vice versa, cellular lipid metabolism affects the assembly and the secretion of lipoproteins into the plasma. Vance and Vance argue that preferential assembly of newly synthesized lipids into lipoproteins acts as the driving force for the secretion of lipoproteins from the endoplasmic reticulum via the Golgi apparatus to the cell surface.

Lipids either taken up or synthesized by cells must be distributed correctly among cellular membranes. The unique lipid composition of each cellular membrane necessitates efficient and well-balanced processes for the supply of membrane lipids to organelles. Routes and mechanisms of lipid transport have been studied in mammalian cells (Voelker), plant cells (Arondel and Kader) and microorganisms (Daum and Paltauf). Biochemical, cytological and molecular biological techniques were em-