Biochimica et Biophysica Acta, 604 (1980) 423–475

BBA 85213

MEMBRANE ASYMMETRY

A SURVEY AND CRITICAL APPRAISAL OF THE METHODOLOGY

II. METHODS FOR ASSESSING THE UNEQUAL DISTRIBUTION OF LIPIDS

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(Received March 24th, 1980)

Contents

I. Summary

In the companion paper, I have reviewed the techniques employed for assessment of the asymmetric distribution and orientation of membrane proteins. This article deals with methods applicable to the investigation of the unequal distribution of lipids between the two membrane leaflets. Among the techniques I will discuss are the use of immunological techniques and lectins, chemical reagents, enzymatic isotopic labeling and degradation of

Abbreviations: SDS, sodium dodecyl sulfate; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*'-tetraacetic acid; TEMPO, 2,2,6,6-tetramethylpiperidine-*N*-oxyl.

membrane lipids, exchange proteins and physical techniques. Whenever appropriate, problems of crypticity and non-availability of lipids to interact with the appropriate ligands, reagents, modifying enzymes or exchange proteins have been envisaged. It appears that in many cases, highly discordant results, sometimes with the same biological material, have been obtained. Some of the difficulties encountered presumably stem from the reported existence of non-bilayer arrangements and isotropic movement of lipids as evidenced by freeze-fracture and NMR studies. Other problems may be related to the induction of such arrangements, especially the inverted micellar arrangement, by the modifying agents, particularly degradation enzymes or exchange proteins when they cause severe unilateral modification of the lipids of the exposed leaflet. In addition, the situation is complicated by the role of the induced increase in the flip-flop rate under different experimental conditions and by modification of the rearrangement of lipid molecules as a result of the metabolic state of the cell or ghost preparation and of the reactivity of lipids as a consequence of temperature changes. Here, more so than with proteins, one must be cautious in interpreting experimental results. Moreover, it would appear that the use of different techniques in conjunction and the consequent comparison of results should be recommended. It has been emphasized that 'general rules' do not hold and that each new material should be assayed again. To give one example, it is not pertinent to state that proteins enhance the flip-flop rate in lipid vesicles (and hence in membranes). This holds true for glycophorin from erythrocyte membrane, but could not be proved when mitochondrial cytochrome oxidase was used.

There seems to be no rule for the distribution of lipids between the two leaflets of different membranes. For example, even for different strains of the same bacterial species, highly divergent results have been reported. It is generally (and probably under the influence of different studies with erythrocytes) believed that in mammalian plasma membranes, choline phospholipids are enriched in the outer leaflet and aminophospholipids in the inner leaflet. Though this contention may prove to be correct, different instances of contradictory results have been given in the text. This shows that if rules do exist, they remain to be discovered or established. Finally, an important question may be raised: does the unequal distribution of lipids, as revealed by the present techniques, always correspond to the best fit for the function of each of the oriented proteins?

II. Introduction

Introductory remarks concerning membrane asymmetry accompanying my previous review articles [1,2] will not be repeated here. Although, in most cases, the asymmetric orientation of proteins reflects an advantage for the cell or, for example, with colicin, phage [3] or lectin receptors [4] may be a property evolved for other purposes [1], the advantages inherent in the unequal distribution of lipids do not appear clearly, except for those parts in contact with integral membrane proteins or enzymes, of which the function may be influenced by lipids and the structure of which has evolved in such a way that they can bind the lipids needed for optimum functioning. An advantage which by itself does not explain asymmetry has been reported in the case of platelets. In this case, accumulation of acidic phospholipids in the inner leaflet of the membrane protects the blood coagulation factors against the coagulation-promoting action of these lipids [5,6]. The asymmetric interaction of drugs categorized as 'crenators' and 'cup formers' with erythrocyte membrane leaflets has also been correlated with the unequal distribution of lipids [7]. Lipopolysaccharides may be of importance to bacteria, protecting them from the

adverse natural conditions of the environment [8]. Sphingoglycolipids may serve as receptors for hormones and also for bacterial toxins such as cholera toxin [9].

In recent reports, the possibility of the transbilayer movement of lipids, particularly in active membranes such as those of bacteria [10,11] and endoplasmic reticulum [12] has been suggested. This movement has been attributed to the presence of proteins [11,13, 14] or to the presence or induction of non-bilayer phases [15,16] (see also Refs. 17–23), these causes not being mutually exclusive. These recent developments shed some doubt on certain reported cases of asymmetry and in fact, as we will see, such contradictory data on lipid distribution in different membranes can be found in the literature, so that a definitive unequal distribution is thought to be shown only in the case of erythrocyte membranes [23]. The unequal distribution of lipopolysaccharides in the external membrane of procaryotic gram-negative bacteria and of glycolipids and staloglycolipids in eucaryotic cells seems to be also generally accepted. However, attention has been legitimately drawn [23] to the most common lipids. Nonetheless, the general occurrence of unequal distribution of lipids, irrespective of the degree, is almost certain in view of factors that tend to disturb an eventually equal distribution [1].

In the companion paper [2], I have reviewed methods used for assessing the asymmetric orientation and distribution of membrane proteins. This article is concerned with the techniques available for assessing the distribution of membrane lipids. I will discuss the use of: immunological techniques and lectins, chemical reagents, enzymatic isotopic labeling and degradation, lipid exchange proteins and physical techniques.

III. Immunological techniques and the use of lectins

Antibodies have been used for the localization of lipopolysaccharides in gram-negative bacteria and have been applied to the study of the lateral mobility of these lipids in the outer membrane of *Salmonella typhimurium* [24,25]. Antibodies were raised against the polysaccharide moiety of lipopolysaccharides and were conjugated to ferritin *. It was observed that at 0°C, the nascent lipopolysaccharides remain immobile, although at 37°C they diffuse away from the 'export site' **. When the peptidoglycan (murein) layer was removed by lysozyme digestion at 0°C, the label was localized in the outer leaflet of the outer membrane. However, if the attack on the murein layer was carried out using lysozyme or if trypsin was used at physiological temperatures (25–37°C), lipopolysaccharides were revealed on both faces of the outer membrane. Thus, a rapid 'rearrangement' of lipopolysaccharides can take place leading to an artificially symmetric distribution of these lipids, despite their unequal, and probably in this particular case, asymmetric distribution. These observations furnish an explanation for reports on the symmetric distribution of lipopolysaccharides revealed previously by the use of ferritin-conjugated antibodies during experiments on penicillin-treated *S. typhimurium* [26,27].

It is worthwhile mentioning with respect to the localization of lipopolysaccharides

* The ferritin-labeling method mentioned here and other methods [2] will not be detailed in this article.

** The use of mutants defective in lipopolysaccharide biosynthesis leads to the observation that the newly synthesized molecules are translocated at a limited number of sites on the bacterial surface and remain in clusters if, shortly (30 s) after the beginning of biosynthesis, cells are cooled. At 37°C they diffuse, arising in an even distribution [25].

that the direct study of anionic sites attributed to carboxyl or phosphate groups of these molecules was performed [28] by using positively charged Fe_2O_3 hydrosols [29] in acetic acid [30] or propionic acid [31] and multiply positively charged ferritin derivatives * [33]. Anionic sites were found distributed uniformly over the entire cell surface [28] of *Brucella canis*. In other studies, large amounts of basic proteins (lysozyme, cytochrome *c*) were found to bind to the cell surface of *Acholeplasma laidlawii* [34], although in *Mycoplasma hominis*, phospholipase C failed to hydrolyse phospholipids in intact cells or isolated membranes, presumably because of the inacessibility of appropriate lipids to the enzyme **.

Antibodies to different classes of lipid have been prepared in order to study their surface exposure. Generally, it was also necessary to use auxiliary lipids such as lecithins and cholesterol in order to demonstrate a reaction between the isolated lipid antigen and its antibodies [41,42]. Similarly, it was necessary to inject methylated serum albumin and auxiliary lipids with lipids in order to prepare antibodies. The polar head groups of phospholipids constitute the antigenic determinants. For example, for both phosphate moieties of cardiolipins, the distance between them and the free hydroxyl of the central glycerol moiety has been found to have an important influence on serological activities [43]. 'Natural' antibodies to phospholipids of *A. laidlawii* were found in guinea-pig serum [44], so that the organism was attacked by fresh serum of non-immunized animals. The classical pathway but not the alternative pathway of the complement system was activated by these antibodies ***.

Antibodies to phosphatidylinositols were raised in rabbits by injecting the phospholipids with an 'adjuvant', namely the auxiliary lipids (phosphatidylcholines and cholesterol). By using these antibodies, 15% of the phosphatidylinositols in myelin and in

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* The preparation of multiply positively charged ferritin is carried out by activating its carboxyl groups with a water-soluble carbodiimide; activated carboxyl groups then react with a nucleophile such as *N,N*-dimethyl-1,3-propanediamine to form tertiary amine-type derivatives or with 1,6-diaminohexane to form primary amine derivatives [32,33].

** The negativity of cell surface charges is often observed by using positively charged colloidal iron or positively charged ferritin derivatives. Colloidal iron has been used to label liver cells [36], erythrocytes [37], chromaffin granules [38] and *Xenopus levis* oocytes [39]. Multiply positively charged ferritin derivatives, which have the advantage of reacting at physiological pH, have been used to label surface sites in erythrocytes, lymphocytes and spermatozoa [33]. The surface sites reacting with positively charged reagents may be sialic acid derivatives, as shown by their sensitivity to neuraminidase [33] and the redistribution of the complexes formed with membrane particles as in the case of erythrocytes [40]. However, with chromaffin granules, although the value of their isoelectric point was estimated to 3.0 [41], neuraminidase treatment did not induce noticeable changes in the pattern of colloidal iron binding [38]. With *X. levis* oocytes [39], treatment with positively charged colloidal iron [29] led to the observation, using electron microscopy, that at the peak of vitellogenic activity, when the microvilli are well developed, negatively charged surface molecules are preferentially localized in the distal portions of microvilli whereas crypts (or invaginations) between microvilli and endocytotic pits are not labeled [39]. In this case, sialic acid of sialoglycoproteins is responsible for retaining iron particles and neuraminidase treatment of oocytes prior to treatment with colloidal iron leads to a marked reduction in the amount of labeling [39]. During the early stage of oogenesis as well as after the peak of vitellogenesis, a random distribution of negatively charged surface molecules exists. Consequently, a redistribution of these molecules takes place during the peak of vitellogenic activity in which molecules synthesized in liver must enter the cell by endocytosis.

*** For a description of the complement system and recent developments, see Refs. 45–48.

microsomal membranes from rat brain were detectable [49]. However, incubation at 45°C increased to approx. 50% the amount of phosphatidylinositols detectable using these antibodies. During these studies, it was observed that synaptic membrane phosphatidylinositols also have a limited capacity to adsorb antibodies. In this case, 'annealing' increased the amount of detectable phosphatidylinositols to only 25%. However, synaptic membranes and mitochondria contain cardiolipins which cross-react with antibodies to phosphatidylinositols, thus hampering quantitative studies. Interpretation of these observations in terms of membrane lipid asymmetry seems uncertain and 'steric, ionic and hydrophobic interactions' have been invoked [49] to explain the fact that acidic lipid antibodies have little capacity to react with the acidic phospholipids of membranes. Studies with mitochondria and microsomes have indicated that adsorption of anti-phosphatidylinositol antiserum is enhanced after proteolytic digestion of these membranes [50], a phenomenon which will be discussed below.

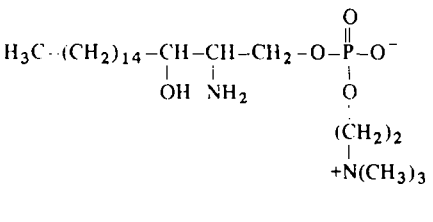
Attempts have been made to examine the antigenic activity of phosphatidylglycerols [51,52]. Lipid extracts of *Mycoplasma pneumoniae* were reported to be responsible for the complement-fixing activity of antisera to *M. pneumoniae* cells. Lipids were found to react specifically and to block antibodies responsible for growth and metabolic inhibition of this organism. However, lipid extracts were found to be unable to elicit an antibody response and, consequently, were regarded as haptens [51]. After fractionation of *M. pneumoniae* lipids, it was found that glycolipids were responsible for specific serological activities, whereas phosphatidylglycerols were inactive, though they could serve as auxiliary lipids enhancing the serological activities of glycolipids. Studies with *M. hominis* seem at variance with these conclusions [52]. Phosphatidylglycerols, which constitute 87% of the phospholipids in this organism, were reported to be immunologically active. Antibodies directed against phosphatidylglycerols were detected in rabbits immunized intravenously with *M. hominis* cells or membranes isolated therefrom, or injected with a flocculated complex of methylated bovine serum albumin and phosphatidylglycerols/phosphatidylcholines/cholesterol. It was observed that antibodies are specifically bound to intact *M. hominis* (as shown by complement-fixation and Coombs' tests). However, cells were not agglutinated unless treated with anti-phosphatidylglycerol antibodies after partial digestion of membrane proteins with pronase. It was concluded that most of the phosphatidylglycerol molecules are masked, probably by proteins.

Mycoplasma mycoides var. *capri* contains immunologically active phosphoglycolipids, presumably identical to glycerophosphoryldiglucosyldiglycerides which are the main components of membrane glycolipids in this organism. Anti-phosphoglycolipid antibodies have been induced [53] by intravenous injection of the flocculated complexes of methylated bovine serum albumin with a mixture of the phosphoglycolipid and the auxiliary lipids (phosphatidylcholines and cholesterol). The antibodies are specific to both the phosphate and the carbohydrate moieties of the phosphoglycolipids used for immunization. Anti-phosphoglycolipid antibodies specifically react with intact organisms and isolated membranes of *M. mycoides*, as demonstrated by complement-fixation and agglutination tests. The antigenic determinants of phosphoglycolipids were found to be mainly located on the outer membrane surface.

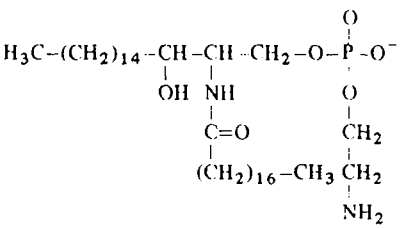
Studies with mitochondria of different origin (rat liver, blowfly flight muscle, *Saccharomyces cerevisiae* and *Neurospora*) showed that only a small percentage of cardiolipins react with anti-cardiolipin antibodies and none of the cardiolipins in intact bovine heart mitochondria were found to be available. It has been observed that cardiolipins from both sides of the mitochondrial membrane do not react appreciably [54];

even removal of F_1-ATPase from inside-out vesicles of bovine heart mitochondria did not unmask the antibody-binding activity. Consequently, cardiolipins were thought to be 'shielded' [54]. However, the interactions of anti-cardiolipin antibodies with mitochondria and the inner mitochondrial membrane [55] and those of anti-phosphatidylinositol antibodies with mitochondrial, inner mitochondrial and microsomal membranes have been observed [55]. It was concluded that the polar head groups in each case are accessible to antibodies, the pronase treatment exposing even more antigenic determinants. The fact that intact mitochondria react with anti-cardiolipin antibodies is explained by the damage caused to the outer mitochondrial membrane, which is impermeable to proteins, during the complement-fixation assay. However, a quantitative estimate of 'buried' and 'free' antigenic head groups was not possible. Recently, anti-cardiolipin antibodies were found to react with inside-out submitochondrial particles [56]. After digestion with trypsin, more antigenic sites were exposed and could interact with the antibodies. The same treatment applied to mitoplasts led to only marginal effects. These studies and those on phospholipase A_2 degradation indicated that the inner leaflet of bovine inner mitochondrial membrane is about 3-times richer in cardiolipins than the outer leaflet of this membrane [56].

Anti-sphingomyelin specific antibodies have been elicited in rabbits by using conjugates of two synthetic analogues of sphingomyelins, dihydrosphingosine phosphorylcholine and ceramide phosphorylethanolamine [57], both bound covalently to either a synthetic polypeptide carrier or to a protein carrier. Though ceramide phosphorylethanolamine conjugates are more potent in producing anti-lipid antibodies, the specificity of the antibodies elicited was not different from those raised against dihydrosphingosine phosphorylcholine derivatives. The synthetic carriers used were either a copolymer of the amino acids, alanine, glutamic acid, lysine and tyrosine (6 : 1.9 : 4.7 : 1), called cop 1 with an average molecular weight of 23 000 or a copolymer of glutamic acid and tyrosine (4 : 1) with an average molecular weight of 42 000. The protein carrier used was bovine serum albumin.



dihydrospingosine phosphorylcholine



ceramide phosphorylethanolamine

For the preparation of conjugates of ceramide phosphorylethanolamine, dicyclohexylcarbodiimide and hydrophobic solvents or a mixture of solvents were used. For coupling serum albumin with dihydrosphingosine phosphorylcholine, a water-soluble carbodiimide (1-ethyl-3-(3-dimethylaminopropyl)carbodiimide) was employed. In each case, the carboxyl groups of glutamic acid residues of either synthetic polymers or the protein were coupled to the free NH_2 group of dihydrosphingosine phosphorylcholine or ceramide phosphorylethanolamine (see the formulae). As mentioned above, auxiliary lipids are generally needed to demonstrate the interaction of a lipid antigen with its antibody. The advantage of the synthetic analogues of sphingomyelins is that as they are water-soluble material, they can react with their antibodies without the need for auxiliary lipids [57]. On the other hand, antibodies with specificity directed towards synthetic derivatives of

sphingomyelins are capable of recognizing the naturally occurring compounds in situ, in the membrane of ovine red blood cells, causing specific hemolysis in the presence of complement. Ceramide phosphorylethanolamine conjugate specifically inhibits this hemolysis. Guinea-pig erythrocytes with a low level of sphingomyelins do not undergo hemolysis. It has been suggested that the antibodies thus prepared may be used for localization of the lipid in tissues and cell membranes [57].

Surface carbohydrates of Mycoplasma, presumably bound to glycolipids, have been studied by using lectins. It was observed that *A. laidlawii* and *Mycoplasma fermentans* are devoid of lectin-binding sites at the cell surface, but that pronase digestion could reveal galactose groups [58]. It was suggested that the glycolipids are hidden under a protein layer. In other cases, such as *M. pneumoniae* and some temperature-sensitive mutants, glycolipids with both galactose and glucose groups were found exposed on the cell surface [58]. However, with the above-mentioned mutants, the lectins of *Phaseolus vulgaris* and *Vicia cracca*, which react with the N-acetylglucosamine group, agglutinated *Mycoplasma gallinarum*, *M. mycoides* var. *capri* and *Mycoplasma pulmonis*. However, the agglutination was lost after pronase treatment, indicating that the carbohydrates are probably protein-bound. With *M. hominis*, neither intact nor pronase-treated cells react with lectins [58]. For the visualization of surface carbohydrates linked to glycolipids in *M. mycoides* var. *capri*, concanavalin A which reacts with a carbohydrate of the α-D-glucosyl or a similar configuration was used. The lectin was bound to the glutaraldehyde-fixed cells in suspension and advantage was taken of the fact that horseradish peroxidase is a glycoprotein to react the enzyme non-covalently with the free valences of the fixed concanavalin A. After reaction with diaminobenzidine, post-fixation is carried out with OsO_4 to reveal the peroxidase (for more details see the companion paper [2] and references therein). The fact that glycolipids are involved was ascertained from pronase treatment which did not change the staining pattern of the cell surface, whereas lipid extraction with acetone abolished completely the cytochemical reaction. The glycolipid involved may be a mixture of glycerophosphoryldiglucosyldiglycerides and small amounts of di- and monoglucosyldiglycerides. In addition, a glucose-containing surface polysaccharide, presumably a glycan, can react with concanavalin A [59].

In other investigations [60], lectins such as wheat germ agglutinin, *Ricinus communis* agglutinin and concanavalin A were iodinated by the chemical method using chloramine T (for details see the companion paper [2] and references therein) and the surface receptors of Mycoplasma cells were studied using the interaction with radioactively iodinated lectins. Mycoplasma cells and leaky membranes were found to bind lectins specifically. Since the binding to whole cells is similar to that of membranes, it was suggested that only sites at the outer surface react. Proteolysis of the cells increased the lectin binding capacity, indicating that additional carbohydrate groups are unmasked [60]. Therefore, these investigations revealed the asymmetric distribution of carbohydrate sites, and as indicated by lectin binding which is more sensitive than the agglutination test used in previous studies [58], all Mycoplasma species have exposed carbohydrate-containing molecules. Since proteolysis generally increased the lectin binding, glycolipids or other protease-resistant sites could be involved. In a few species of Mycoplasma, a decrease in binding occurred after proteolysis, indicating that glycoproteins are involved or that rearrangements took place [60].

IV. Chemical reagents

Most reagents used for assessing the asymmetric orientation of proteins can be employed in studying lipids. Experiments performed by Maddy [61] with erythrocytes using 4-acetamido-4′-isothiocyanostilbene-2,2′-disulfonate led to the observation that aminophospholipids, phosphatidylethanolamines and phosphatidylserines were not labeled by this reagent. The reason for this non-reactivity is that these lipids are mostly not exposed to the external surface of erythrocytes and that this reagent is not permeant. The use of formylmethionyl(sulfone)methyl phosphate by Bretscher [62,63] demonstrated the asymmetric distribution of phospholipids in the erythrocyte membrane. Imidoesters have also been used for the study of lipid distribution. When erythrocytes and their ghosts were completely amidinated by penetrating and non-penetrating imidoesters, the permeant analogue modified the aminophospholipids in the same proportions in the intact cell and in non-sealed ghosts and, as expected, the non-penetrating reagent modified ghosts to a larger extent [64]. As discussed more extensively in the companion paper [2], the permeant analogue used was ethylacetimidate and the non-permeant analogue isethionylacetimidate.

Trinitrobenzenesulfonate has been a widely used reagent for assessing aminophospholipid distribution in membranes of different origin such as erythrocytes [65–67], platelets [68], murine fibroblast (LM) cells [69,70], normal and virus-transformed hamster embryo fibroblasts [71], bacteria [10,11,14,66,72–77], viruses [78,79], sarcoplasmic reticulum [80,81], mitochondria [67], retinal rod outer segment disc membranes [82, 83], synaptosomes [84–86] and liposomes [76,82,87]. In addition to trinitrobenzenesulfonate, other amino group reagents such as 1-fluoro-2,4-dinitrobenzene and 1,5-difluoro-2,4-dinitrobenzene have been used. The latter compound is a bifunctional cross-linking reagent [66,67]. These reagents can be used for lipids and proteins. However, though trinitrobenzenesulfonate is regarded as a non-penetrating reagent because of its charged sulfonic group, fluorodinitrobenzene and difluorodinitrobenzene do penetrate the hydrophobic core of membranes. Assays with trinitrobenzenesulfonate and fluorodinitrobenzene showed that, in the presence of $NaHCO_3$ buffer at pH 8.5, the reaction of phosphatidylethanolamine and phosphatidylserine dispersions goes to completion [66,67]. Phosphatidylethanolamines and phosphatidylserines from sonicated dispersions of red blood cell lipids also react to completion with fluorodinitrobenzene. Isolated phosphatidylethanolamines and phosphatidylserines react to completion with trinitrobenzenesulfonate in a $CHCl_3/CH_2OH$ (1 : 1, v/v) mixture (1 ml) containing 0.1 ml of 5% $NaHCO_3$ buffer. Phosphatidylethanolamines react to completion within 20 min and phosphatidylserines within 60 min [65]. It is interesting to note that studies with albumin have shown that 26–30% of the amino groups of this protein are accessible to trinitrobenzenesulfonate [88]. The heterogeneity of the amino groups of serum albumin in the reaction with trinitrobenzenesulfonate has been reported [89]. Some authors demonstrated the penetration of trinitrobenzenesulfonate through the erythrocyte membrane; this is probably due to the presence of the band 3 protein which is an anion transporter with a very broad specificity [90,91]. As we will see, differential behavior of trinitrobenzenesulfonate, depending on temperature, has also been reported. Using trinitrobenzenesulfonate, Gordesky and Marinetti [65,92] and Marinetti and Love [66] observed that 20% of the phosphatidylethanolamines of erythrocytes can react. In a rare genetic abnormality, human erythrocytes (En(a$^-$) erythrocytes) lack glycophorin A [93] and the heterozygous erythrocytes (En(a) erythrocytes) contain half the normal amount of this major sialoglycoprotein. The amount of aminophospholipids was found to be normal in

all erythrocyte variants. It was observed that a relatively large amount of these lipids, 23 and 26%, reacted with trinitrobenzenesulfonate in En(a) and En(a$^-$) cells, respectively [94]. Whether these results indicate the enrichment of the outer leaflet of the 'unmasking' of a certain amount of phosphatidylethanolamines, due to the absence of the major sialoglycoprotein, remains to be elucidated. We will see that glycolipids are also more easily labeled in En(a$^-$) cells than in normal cells.

The use of trinitrobenzenesulfonate for the evaluation of lipid sidedness in human platelets showed that only 12 - 18% of the phosphatidylethanolamines were accessible and that the phosphatidylserines were not labeled [68]. In a murine cell line (LM cells), trinitrobenzenesulfonate reacted with only 4% of the phosphatidylethanolamines and 5% of the phosphatidylserines. Unsaturated fatty acid chains and long chains were preferentially localized in the inner leaflet. When isethionylacetimidate was used, 6% of the phosphatidylethanolamines were labeled. The permeant reagent, methylacetimidate, labeled more than 80% of the phosphatidylethanolamines [70]. These results do not seem to be in good agreement with observations made on the same cell line which indicated 70% labeling by trinitrobenzenesulfonate of phagosomal phosphatidylethanolamines and 24% labeling in phagosomes prepared from prelabeled cells [69]. In the latter study, about 52% of the phosphatidylcholines were found to be readily exchangeable from latex phagosomes, indicating the equal distribution of this lipid in both leaflets [69] (see also below).

Analysis of lipids of normal and virus-transformed hamster embryo fibroblasts showed a decrease in phosphatidylcholines and phosphatidylethanolamines but a marked increase in a phospholipid, provisionally identified as phosphatidylthreonines, in transformed cells [71]. Phosphatidylethanolamines of transformed cells were found to react to a greater extent with both trinitrobenzenesulfonate and fluorodinitrobenzene, but phosphatidylserines and phosphatidylthreonines of both normal and transformed cells did not react with trinitrobenzenesulfonate. Normal cells contain only traces of phosphatidylthreonines, but in transformed cells both compounds existed in the same proportions. Trinitrobenzenesulfonate labeled only 11.8% and 18.8 ± 12% of the phosphatidylethanolamines in normal and transformed cells, respectively; but the permeant reagent, fluorodinitrobenzene, labeled 64% and 88% ± 14% of the phosphatidylethanolamines in normal and transformed cells, respectively. Furthermore, trinitrobenzenesulfonate did not react at all with either phosphatidylserines or phosphatidylthreonines, whilst fluorodinitrobenzene reacted with 8.2 ± 6% of the phosphatidylserines of normal cells and with 10.3 ± 3% of phosphatidylserines or phosphatidylthreonines of transformed cells. Despite the greater accessibility of aminophospholipids in transformed cells, phosphatidylthreonines were primarily labeled. Phosphatidylserines were thought to be more tightly bound to proteins. Essentially none of the phosphatidylserines or the phospholipid identified as phosphatidylthreonines are exposed to the outer leaflet of normal and transformed hamster embryo cells [71].

By means of labeling using trinitrobenzenesulfonate and isethionylacetimidate, the distribution of phosphatidylethanolamines in *Bacillus megaterium* membrane has been examined. On average, 33% of the total phosphatidylethanolamines could be modified. Using inverted vesicles and trinitrobenzenesulfonate, it was possible to label the remaining phosphatidylethanolamines. It was concluded that phosphatidylglycerols, the other major phospholipids of *B. megaterium*, are localized essentially in the external leaflet [10]. In this experiment, Rothman and Kennedy [10] observed that trinitrobenzenesulfonate does not penetrate the *B. megaterium* membrane at 0°C. However, at 15°C, the reagent

slowly penetrates the cell membrane [10]. Fong et al. [78] used trinitrobenzenesulfonate to study the unequal distribution of phosphatidylethanolamines in the membrane of vesicular stomatitis virus. They found that, although disruption of the membrane by sonication rendered all the phosphatidylethanolamines reactive to trinitrobenzenesulfonate, this reagent labeled only 36% of this lipid in viruses or trypsin-treated viruses. Therefore, 36% of the phosphatidylethanolamines were attributed to the outer and 64% to the inner leaflet of the viral membrane. Trypsinization was performed to unmask lipids hidden by the glycoprotein of the viral envelope. These results have been confirmed in more recent studies on vesicular stomatitis virus grown on monolayer culture of baby hamster kidney (BHK-21) cells. In addition, it was observed that the inner leaflet phosphatidylethanolamines contain a significantly higher proportion of unsaturated fatty acyl chains. In the outer leaflet, position 1 of the *sn*-glycerol moiety of phosphatidylethanolamines is enriched in saturated acyl chains, containing more than 60% of saturated chains, while in the inner leaflet approx. 30% of the chains at position 1 are saturated. Position 2 of the *sn*-glycerol moiety has the same composition with regard to the proportion of unsaturated chains. This was one of the first instances in which a difference in fatty acyl chain composition between pools of the same phospholipid on opposite membrane leaflets was reported [79]. Other studies [18] have demonstrated the unequal distribution of unsaturated chains, and now more examples are accumulating [70] (see also below). Vale [80] used a combination of trinitrobenzenesulfonate and fluorodinitrobenzene in studying the distribution of aminophospholipids in sarcoplasmic reticulum. Other studies have confirmed that phosphatidylethanolamines are preferentially located on the outer leaflet and phosphatidylserines on the inner leaflet, phosphatidylcholines and lyso derivatives being equally distributed [81]. Combination of the reaction of trinitrobenzenesulfonate and fluorodinitrobenzene with mitoplasts and electron-transferring (ETP_H) mitochondrial particles led to the conclusion that 65% of the phosphatidylethanolamines of mitoplasts are localized on the outer leaflet while 66% of the reactive amino groups of protein are localized on the inner leaflet [67].

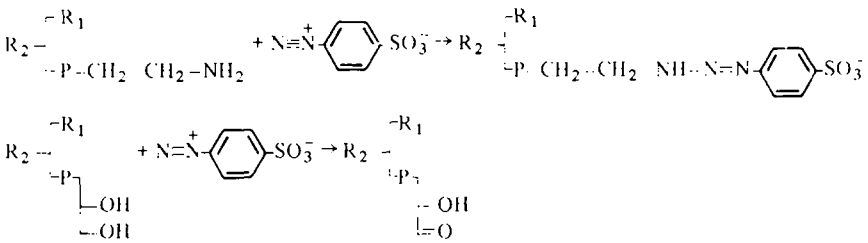
Studies by Litman [82] and Litman and Smith [83] with bovine retinal rod outer segment disc membranes using trinitrobenzenesulfonate showed that phosphatidylethanolamines are preferentially located on the outer surface of the discs while phosphatidylserines are preferentially located on the inner surface.

Murine brain synaptosomal plasma membrane was treated under 'non-penetrating conditions' with trinitrobenzenesulfonate and about 10–15% of the phosphatidylethanolamines and 20% of the phosphatidylserines of the outer membrane were labeled. In addition, externally exposed phosphatidylethanolamines (labeled molecules) were richer in saturated fatty acids [84,85]. These results are in contrast with the previously reported distribution of lipids in synaptosomal plasma membranes and isolated synaptosomal membranes which locate most phosphatidylserines and greater than 50% of the phosphatidylethanolamines on the outer leaflet [86]. These experiments were performed on brain preparations using ^{3}H-labeled pyridoxal phosphate as the amino group reagent. Pyridoxal phosphate does not penetrate the membrane, since, when synaptosomes were labeled and then the synaptosomal membrane isolated, the soluble protein fraction was not labeled. With phosphatidylserines, the extent of labeling was found to be the same in synaptosomes and in isolated membranes. It was concluded that most or all of this phospholipid is probably externally located. With phosphatidylethanolamines, the extent of labeling in synaptosomes was only half that in synaptosomal membranes. It was suggested that some of this lipid may be on the inner surface or that, alternatively, it may all be

externally oriented but less accessible to reagent in the intact synaptosomes [86].

Further comment seems appropriate concerning the widely used reagent, trinitrobenzenesulfonate. Incomplete labeling of aminophospholipids with this reagent has been observed [65,66,75,80]. It has indeed been reported that under conditions in which both sides of *Bacillus subtilis* cytoplasmic membrane are exposed to this reagent, only 60–70% of the total aminophospholipids react [75]. Under the same conditions, however, complete hydrolysis of phospholipids by phospholipase C from *Bacillus cereus* can be achieved. On the other hand, when trinitrobenzenesulfonic acid was injected under a monolayer of aminophospholipids, it was observed that only 80% of the phosphatidylethanolamines of *B. subtilis* could be converted into the trinitrophenyl derivatives. When cardiolipins were a component of the monolayer, a 20% decrease in the extent of trinitrophenylation of phosphatidylethanolamines was observed. The trinitrophenylation gave rise to a significant increase in the surface pressure and the reaction did not proceed to completion. The bulkiness and the charge of trinitrobenzenesulfonate have been invoked to explain the failure to achieve complete labeling [23,75,95]. However, the fact that 10% of the phosphatidylethanolamines in sarcoplasmic reticulum are not labeled by trinitrobenzenesulfonate was explained on the basis of their strong interaction with proteins [80].

Diazosulfanilate has been used for the study of the distribution of lipids in PM_2 bacteriophage in which phosphatidylethanolamines and phosphatidylglycerols constitute 95% of the envelope lipids. (These compounds were reported to give, respectively, an azoamino derivative and an aldehyde.) The result of the reaction of the diazonium salt and the phosphatidylglycerols is the oxidation of the free glycerol residue to the monoaldehyde of the glycerol. This aldehyde is then reduced by $NaB[^3H]H_4$ to give the labeled molecule [96].



Using diazosulfanilate and a complex of fluorescamine [2] with cycloheptaamylose, the distribution of aminolipids in sarcoplasmic reticulum [80,81] has been confirmed by Hidalgo [97]; significantly, 70–80% of the phosphatidylethanolamines were located at the external side whereas phosphatidylserines were not accessible to the reagents. After fluorescamine-cycloheptaamylose treatment, both the ATPase activity of sarcoplasmic reticulum Ca^{2+}-ATPase and calcium transport were found to be inhibited. However, vesicles retained their impermeability to inulin [81,97]. It was observed that if partial labeling of 'highly reactive' amino groups with the fluorescamine-cycloheptaamylose complex is performed, then enzyme phosphorylation or Ca^{2+}-ATPase activity is not affected, although calcium transport is inhibited. In these experiments, the rapid labeling of phosphatidylethanolamines, paralleling the time course of Ca^{2+} transport inhibition, led to the suggestion that the modification of this class of lipid might be the cause of the inhibition of Ca^{2+} transfer to the inside of vesicles, while the phosphorylation of the enzyme and the subsequent lysis of the phosphate formed could still occur. However, the possible

involvement of a rapidly reacting amino group of the ATPase itself, involved in Ca^{2+} transfer, has not been excluded [97].

A recent work essentially confirms and extends results on the unequal distribution of lipids in mitochondria [67]. In this study, a combination of fluorescamine labeling, phospholipase A_2 degradation and reaction with antibodies to cardiolipins [56] was used. It was noted that fluorescamine labeled 95% of the phosphatidylethanolamines of the deoxycholate-solubilized bovine heart inner mitochondrial membrane. 41% of mitoplast and 61% of submitochondrial (inside-out) vesicle phosphatidylethanolamines were labeled. In this study, mitoplasts were prepared according to the procedure described previously [98,99] and advantage was taken of the fact that the binding site of cytochrome *c* is located on the intermembrane ('cytoplasmic') side of the inner mitochondrial membrane. Cytochrome *c* was covalently linked to Sepharose 4B as performed before [100]. The preparation of submitochondrial particles was then passed through the column packed with the linked cytochrome *c*, the inside-out vesicles being recovered in the void volume and the right-side-out vesicles retained on the column. I have discussed in the companion paper [2] another method for obtaining orientationally pure submitochondrial particles [101].

V. Enzymatic isotopic labeling of membrane lipids

Essentially two enzyme-labeling methods, namely the lactoperoxidase-$^{125}I^-$-labeling method and the galactose oxidase-NaB[^{3}H]H_4 method, are used. In lipid-distribution studies, the use of the lactoperoxidase-$^{125}I^-$-labeling method, previously thought to label specifically only proteins, has been recently advocated [102–105]. Labeling of lipids, to some extent, was previously observed [106–109]. Labeled lipids have been observed to contaminate acid-precipitated labeled proteins from radioactively labeled cells [109]. The mechanism of lipid labeling remains uncertain but lipid peroxidation, as measured by means of the malondialdehyde content, was shown to occur during enzymatic iodination of rat liver endoplasmic reticulum which led to the loss of cytochrome *P*-450; butylated hydroxytoluene could prevent lipid peroxidation and cytochrome *P*-450 was preserved as a consequence [110]. In the surface protein labeling of intact myelin sheaths, a large amount of label was found to be associated with most lipid classes [108]. If lactoperoxidase was omitted the same amount of radioactivity was incorporated into the lipids, so that the enzyme dependence of lipid labeling could not be confirmed. However, work by Mersel et al. [103] on phosphatidylcholine and phosphatidylcholine/cholesterol liposomes has shown the labeling of lipids in an enzyme-dependent manner. Similarly, not only proteins but also lipids from ovine embryo secondary culture cells have been iodinated by using the lactoperoxidase-$^{125}I^-$-labeling method [103]. In these studies, the labeling of lipid was efficient and in the cell 20–30% of the radioactivity was found in proteins and 20–30% in lipids. Both neutral and polar lipids were labeled. Omission of lactoperoxidase reduced the labeling of liposomes to 6% and that of cells to 2% of that in the presence of the enzyme. Phosphatidylcholines, sphingomyelins, phosphatidylethanolamines, phosphatidylinositols and cholesterol were found to be iodinated. When cells were disrupted by three cycles of freeze-thawing, the amount of label incorporated into the lipids increased 2.5-fold, but in intact cells only the surface lipids were iodinated [103].

The lactoperoxidase-$^{125}I^-$-labeling technique has also been applied to *A. laidlawii* [104]. The distribution of phosphatidylglycerols in this organism was studied using phospholipase A_2. However, Mycoplasma species contain mostly other lipids [105], phospho-

glycolipids and glycolipids which are not attacked by phospholipase A_2. Besides phosphatidylglycerols and diphosphatidylglycerols, phosphoglycolipids (i.e., glycerophosphoryldiglucosylglycerides and glycerophosphorylmonoglucosyldiglycerides) and glycolipids (i.e., monoglucosyldiglycerides and diglucosyldiglycerides) are found in Mycoplasma. Thus, in *A. laidlawii*, phosphatidylglycerols constitute only about 30% of the polar lipids (for details and especially the variability of the ratio of these lipids, see Refs. 104 and 105). Chemical reagents specific for amino groups cannot be used on this organism which lacks aminophospholipids. Radioactive iodination has been suggested as a general labeling procedure for lipids. With *A. laidlawii*, using the lactoperoxidase-$^{125}I^-$-labeling technique, 5% of the radioactivity was found in membrane lipids. 98% of the labeling was enzyme dependent and in a reaction mixture in which $^{125}I_2$ replaced $^{125}I^-$, the labeling of membrane lipids was reduced by 95%. Comparison of the labeling of intact cells (which remain apparently intact) and isolated leaky membranes showed that the extent of labeling of phospholipids and phosphoglycolipids was approximately twice as great in leaky membranes. Thus, phospholipids and phosphoglycolipids are almost equally distributed between the two membrane leaflets. However, the extent of labeling of the two major glycolipids was almost the same in intact cells and isolated membranes. Glycolipids are therefore concluded to be preferentially located on the outer half of the bilayer. The distribution of iodine label was found to be the same at 0, 22 or 37°C. Twice as much lipid was estimated to be in the outer half of *A. laidlawii* membranes as compared to the inner half. Conversely, two-thirds of the membrane proteins face the cytoplasmic compartment.

The nature of the iodine binding site is not yet known. Iodination of the double bonds of chains and iodination of the α-position of carbonyl compounds have been discussed [104]. The latter position was favored, since an increase in saturated chains to 80 mol% of the total fatty acids on growing the cell in the presence of palmitic acid did not affect markedly the level of ^{125}I bound to membrane lipids. On the other hand, highly unsaturated carotenoids were not labeled and among neutral lipids glycerides were labeled rather than cholesterol.

Recently, this technique was used in a study of humoral membrane attack by antibody plus complement. Tumor cells (line-10 tumor cells) were labeled, and 15 min after addition of the complement to the antibody-sensitized cells, specific release of some lipid classes from the cell surface occurred [111].

The galactose oxidase-NaB[^{3}H]H_4 method of labeling which has been used for galactoproteins also labels galactolipids. Glycoproteins or glycolipids endowed with galactose or galactosamine residues at their non-reducing hydroxymethylene end are oxidized catalytically to an aldehyde by galactose oxidase isolated from *Dactylium dendroides*. Oxidation goes no further than the aldehyde [112]. The enzyme cannot penetrate the cell membrane, consequently, only surface-exposed molecules are labeled upon reduction of the aldehydes formed to the corresponding alcohols (by NaB[^{3}H]H_4) [112–115]. Studies conducted on erythrocytes and comparison with results obtained with immunological techniques revealed the crypticity of certain glycolipids. By crypticity, it is meant that a component is exposed to a given membrane surface, but that the reagent cannot reach and hence react with it because of the steric hindrance due to other molecules on that surface. In erythrocytes, glycolipids are essentially glycosphingolipids, i.e., oligohexosylceramides and gangliosides. The most abundant oligohexosylceramides are trihexosylceramides and particularly tetrahexosylceramides (globosides). Glycosphingolipids with A and B blood group activity are also exposed to the surface. In human erythrocytes,

oligohexosylceramides with six to ten (or more) sugar units are also present in very small amounts [116]. Antisera to the latter glycolipids react with erythrocytes; however, antisera to tetrahexosylceramides do not react whilst either fetal erythrocytes or trypsinized erythrocytes react with these antisera. In fetal erythrocytes, the surface is believed to be 'incompletely coated' and in adult erythrocytes trypsin treatment is believed to unmask the globosides by eliminating fragments of glycoprotein. These facts together with the observation that the Forssman antigen, a pentahexosylceramide, does not react with its corresponding antibodies indicated that at least six sugar units are necessary for the accessibility of antibodies [117].

Oligohexosylceramide structures all possess a galactose or an *N*-acetylgalactosamine residue at the terminal end remote from the ceramide moiety of the molecule. Therefore, they can be oxidized using the galactose oxidase-NaB[^{3}H]H$_4$ method. When this was carried out [115], it was observed that tetrahexosylceramides were the glycolipids predominantly labeled. Trihexosylceramides were also found to be reactive, unlike dihexosylceramides. Glycoproteins of 95 000, 82 000 and 64 000 daltons were observed to be labeled at the same time. After proteolytic treatment of erythrocytes, the specific activity of galactosamine in glycolipids and that of galactose in glycoproteins increased, but the total activity of glycoproteins did not change. The labeling of glycoprotein was greatly enhanced by neuraminidase treatment but that of glycolipids less so [115]. The surface exposure of globosides has been doubted since they are not accessible to their relevant antibodies (molecular weight approx. 200 000 [118,119] and since jackbean β-*N*-acetylhexosaminidase (molecular weight approx. 100 000) does not hydrolyse them in erythrocyte membranes, although it is active when an aqueous solution of these oligohexosylceramides is used [115]. However, globosides become reactive with anti-globoside antibodies after treatment of erythrocytes with neuraminidase or a protease [120]. An important change in the labeling of glycolipids is observed upon ghost preparation, since 25% of the globosides of erythrocytes, as compared to 90% in ghosts, are labeled using this technique. The treatment of red blood cells with alkaloids such as cytochalasin B or vinblastine, which disrupt intracellular cytoskeletal elements [120–122] or hinder their assembly, renders 100% of the tetrahexosylceramides and trihexosylceramides reactive. However, the dihexosylceramides remain refractory. This example shows that immunological and enzyme-labeling techniques give different results, probably since the reactive site of the former has less access to surface molecules.

That erythrocyte glycolipids are oriented to the outside, remote from the cytoplasmic compartment, is demonstrated by the observation that no glycolipid is labeled when reversed vesicles are used [114].

As reported by Gahmberg et al. [93], in En(a$^-$) cells, the total sialic acid content of erythrocytes is greatly reduced since PAS1 is absent [93] *. After treatment with neuraminidase, which eliminates sialic acid residues, galactose oxidase activity in PAS2 is reduced due to the absence of the PAS1 monomer which is suggested to form a complex with the genuine PAS2 monomer in the membrane. If labeling using lactoperoxidase and ^{125}I$^-$ is carried out, PAS2 is not iodinated; its tyrosine residues must be more deeply embedded in En(a$^-$) cells; PAS3 is only poorly iodinated [93].

* Erythrocytes contain sialoglycoproteins and sialoglycolipids. There are four sialoglycoproteins, termed PAS1, PAS2, PAS3 and PAS4, which give positive periodic acid Schiff reactions. PAS1 is the sialoglycoprotein called glycophorin, a transmembrane protein which carries A, B and MN antigens as well as receptors for influenza virus, encephalomyelitis virus and phytohemagglutinins [123–125].

Though the sialic acid content of erythrocytes is greatly decreased in En(a$^-$) cells, the total carbohydrate content of all cells remains similar. Band 3 protein contains twice the normal amount of carbohydrate and is enriched in galactose and mannose. This enrichment is explained on the basis of the excess glycosylation of the band 3 protein as a result of the absence of PAS1, which normally competes for the transferase. The excess glycosylation was suggested to prevent the reaction of the tyrosine residue of PAS2 and PAS3 due to steric hindrance. However, although no difference in the composition of the glycolipids was found between different membranes, the content of glycolipid label in En(a$^-$) and En(a) heterozygous cells is appreciably greater than in normal cells. The explanation furnished is that normally the major sialoglycoprotein partially impedes the labeling of glycolipids. Nonetheless, protease treatment of normal cells which removes sialoglycoproteins [123,126] does not change the amount of label in glycolipids to the extent found in En(a$^-$) and En(a) cells.

Crypticity has been observed with sialoglycolipids of rat brain synaptosomes. Here, the galactose oxidase-NaB[^{3}H]H$_4$ method labeled mostly gangliosides GM_3. 70% of the label was found on these gangliosides. With isolated membranes of the synaptosome, however, gangliosides GM_2, GD_2 and GD_1 were also appreciably labeled [127].

VI. Enzymatic degradation of membrane lipids

Enzymes which are capable of cleaving lipids can be used, since, in principle, these enzymes do not penetrate the membrane and should react with the molecules of the exposed leaflet (see, however, below). The action of sialidase (neuraminidase, *N*-acetylneuraminidate glycosyltransferase) on erythrocytes eliminates the sialic acid units from all sialic acid-bearing proteins or lipids [128]. However, with tumorigenic cells such as murine melanoma B16 cells, although isolated sialolipids are degraded by *Vibrio cholerae* enzyme, a Ca^{2+}-requiring sialidase, it has been shown that the enzyme attacks protein-bound but little if any lipid-bound sialic acid residues of sialolipids, comprised mostly of hematosides (sialyllactosylceramides). Recently, Barton and Rosenberg [129] reported that 30% of the sialolipids were degraded to lactosylceramide by the action of *Clostridium perfringens* sialidase, which is indifferent to the presence of Ca^{2+} in the medium, although the cleavage of sialic acid units from sialoglycoproteins requires Ca^{2+}. This example demonstrates that an enzyme with an approximate molecular weight of 60 000 does not have access to all the sialic acid residues of surface molecules, especially those of sialolipids. Sialidase has found other applications, in particular with vesicular stomatitis virus. It has been observed that the total content of *N*-acetylneuraminic acid present in the hematosides of the envelope is hydrolyzed on treatment with sialidase, though the virion retains its morphology on electron micrographs. The neuraminic acid free virions showed a strong tendency to aggregate [130,131]. These results clearly show the sidedness of sialoglycolipids in vesicular stomatitis virus.

Phospholipases are the most widely used enzymes in assessing the distribution of membrane lipids. As soon as the unequal distribution of phospholipids in erythrocyte membrane leaflets was revealed by chemical labeling [61–63], Zwaal et al. [132] refined and adapted their work on enzyme degradation. In contrast to most other techniques which concern specific lipid classes, all lipid components can be attacked by a judicious choice of enzymes. When using phospholipases to attack membranes, the intrinsic specificity of these enzymes should be borne in mind. For example, *B. cereus* phospholipase C is only active on glycerophospholipids and is inactive on sphingomyelins [133,134]. *C. per-*

fringens (previously called *Clostridium welchii*) phospholipase C is inactive on phosphatidylglycerols, cardiolipins, phosphatidylserines [133,135] and phosphatidylinositols (see below), but acts on sphingomyelins [133,135–137]. *Staphylococcus aureus* phospholipase C acts only on the sphingomyelins among the major membrane phospholipids, but is also active on lysophospholipids [137,138]. Cardiolipins are poor substrates for phospholipase A_2 [139,140]. Furthermore, the procedure used for purification of phospholipase A_2 has been reported to be important [56]. Purified according to the procedure described by Deems and Dennis [141], the enzyme from *Naja naja* is freed from a contaminating 'lytic factor' [56,142]. This factor is believed to cause lysis of the membrane so that the enzyme obtains access to both sides. Another remarkable fact is the reversal of specificity of this enzyme, depending on whether an individual phospholipid is being assayed separately in Triton X-100 mixed micelles or as a mixture of phospholipids in the same detergent-mixed micelles. Thus, individually, phosphatidylcholines are the preferred substrates and in mixed phospholipids phosphatidylethanolamines are preferred [143, 144].

Applied to erythrocytes, phospholipase A_2 from *N. naja* degraded 70% of the phosphatidylcholines whereas neither phosphatidylethanolamines nor phosphatidylserines were attacked. On the other hand, sphingomyelinase of *S. aureus* degraded 80% of the sphingomyelins. Phosphatidylethanolamines and phosphatidylserines are exposed to the cytoplasmic leaflet, since, when erythrocyte membranes are lysed and then treated with phospholipase A_2, all glycerophospolipids are converted to lyso derivatives [132]. Enzymes have been entrapped in resealed ghosts. It was observed that when pancreatic phospholipase A_2 was used 25% of the phosphatidylcholines, half of the phosphatidylethanolamines and about 65% of the phosphatidylserines could be hydrolyzed before lysis occurred [145]. In other experiments, the action of phospholipases A_2 and C on inside-out sealed ghosts was examined. It was found that virtually all phosphatidylethanolamines and phosphatidylserines and about 30–40% phosphatidylcholines and sphingomyelins are susceptible to hydrolysis [146].

Significant observations were made when the basic phospholipase A_2 from a snake (*Agkistrodon halys blomhofii*) venom was used to study hydrolysis of human erythrocyte phospholipids [147]. Two stages were recognized; firstly (stage a), about 70% of the phosphatidylcholines were hydrolyzed without hemolysis; then (stage b) complete hydrolysis of the remaining phosphatidylcholines followed by extensive hydrolysis of phosphatidylethanolamines occurred and, finally, with the onset of hemolysis, attack on phosphatidylserines was noted. Adjustment of experimental conditions led to the observation that at pH 7.4 and low (10 mM) Ca^{2+} concentrations, only the first stage occurred. At pH 8 and/or higher (40 mM) Ca^{2+} concentrations, both stages took place. It was suggested that stage b is mediated by the influx of Ca^{2+} into the cell when ATP levels are low. In fact, addition of glucose (regeneration of ATP) under any of the above-mentioned conditions prevented the occurrence of stage b (i.e., hemolysis) and the progress of phospholipid lysis further than stage a [147]. Recent experiments have demonstrated that spectrin SH oxidation results in the accessibility of 50% of the phosphatidylethanolamines and of 30% of the phosphatidylserines to hydrolysis with phospholipase A_2. This suggests a role for this peripheral, cytoskeletal protein in maintaining the distribution of these lipids [148,149].

Applied to porcine platelets, the enzymatic degradation, including the action of phospholipase A_2 from *N. naja*, has shown that 46% of the total phospholipids exist in the external leaflet and that this leaflet comprises 90% of the sphingomyelins, 40% of the

phosphatidylcholines, 34% of the phosphatidylethanolamines and only 6% of the phosphatidylserines of the membrane, thus indicating the unequal distribution of the lipids [6,150].

As mentioned above, labeling of aminophospholipids using trinitrobenzenesulfonate indicated that 12–18% of the phosphatidylethanolamines were accessible and that phosphatidylserines were not labeled when intact human platelets were used [68]. These results are at variance with the observation of other authors who used phospholipase C from *B. cereus* for assessing the sideness of lipids in human platelets [151] and concluded that intact cells lost about 50–75% of the phosphatidylethanolamines, 20–50% of the phosphatidylcholines and 20–25% of the phosphatidylserines. This enzyme does not hydrolyze sphingomyelins. Neither phospholipase C from *B. cereus* nor sphingomyelinase alone promoted platelet aggregation. The use of these enzymes together resulted in aggregation. The loss of at least 45% of the total phospholipids under the action of phospholipase C was not sufficient to induce aggregation, release reaction or significant impairment of platelet responsiveness to ADP, thrombin or collagen. Phospholipase C from *C. perfringens*, having a different substrate specificity and hydrolyzing, in particular, sphingomyelins, has been shown to induce release reaction [151]. Other studies on platelets have been carried out [152–156] and, recently, bee venom phospholipase A_2 and *S. aureus* sphingomyelinase were used to study human platelets. 45% of the plasma membrane phospholipids were hydrolyzed, comprising 93% of the sphingomyelins, 45% of the phosphatidylcholines, 9% of the phosphatidylserines, 16% of the phosphatidylinositols and 20% of the phosphatidylethanolamines. Futhermore, it was found that the outer leaflet is deficient in arachidonic acid, since only 10% of the cytoplasmic membrane content of this acid was found in this leaflet [154].

Studies of the asymmetric distribution of lipids in subcellular liver cell membrane fractions led to very puzzling and controversial results. Nilsson and Dallner [155,156], using *N. naja* phospholipase A_2, examined the distribution of lipids in microsomal, Golgi, inner mitochondrial, lysosomal and nuclear membrane subfractions. In general, about 50–55% of the total phospholipids were hydrolyzed. 50–60% of the phosphatidylcholines were degraded in different fractions, therefore, their distribution seems to be non-preferential. However, phosphatidylserines present in microsomes, the Golgi fraction and nuclear membrane were almost completely hydrolyzed. Phosphatidylethanolamines behaved similarly to phosphatidylserines. Conversely, 80–90% of the phosphatidylinositols of all particles as well as 82% of the cardiolipins of the inner membrane of mitochondria were not hydrolyzed by phospholipase A_2. It has been observed that pronase treatment does not affect the results. Therefore, if masking of lipids exists it is not relieved by this treatment [156].

Sundler et al. [157] have studied the distribution of lipids in liver microsomal and Golgi fractions. These authors used *Crotalus atrox* phospholipase A_2 and a phosphatidylinositol phosphohydrolase isolated from a crude *B. cereus* phospholipase C. Preliminary experiments with bilayer phospholipid vesicles indicated that 80% of the outer leaflet phospholipids can be degraded without attack on the inner surface. In microsomal vesicles, approx. 40% of the outer surface phospholipid can be lysed before the enzyme gains access to the internal leaflet. By following the degradation of the major phospholipids in intact microsomes and in extracted lipids, it was concluded that the same fraction of each class of lipid is exposed on both sides of the membrane. The same conclusion was reached using Golgi vesicles (the presence of phosphatidylcholine-enriched particles in these vesicles being taken into account [157]). The reason for the discrepancy between

the two sets of results is not clear. As Palade and Siekevitz [158] have shown, microsomal vesicles are released from endoplasmic reticulum without inversion [159,160], as shown by the presence of ribosomes attached to rough microsomes, cytochemical localization of glucose-6-phosphatase [159] and immunocytochemical localization of cytochrome b_5 [160]. Moreover, microsomes can be obtained as a reasonably pure fraction [161]. Thus, perturbation should not be brought about by the process of homogenization. Sundler et al. [157] have suggested that the specificity of the phospholipase A_2 (from *N. naja*) used by Nilsson and Dallner [155,156] may be the cause of the discrepancy. Yet, Higgins and Dawson [162] have obtained other diverging results and stated that 'Nilsson and Dallner have reported a completely reversed distribution of phospholipids to our findings' [162]. These authors [162], used *C. perfringens* phospholipase C and found that 50% of the phospholipids of rat liver microsomal membrane were hydrolyzed without loss of secretory proteins (i.e., damage to these vesicles). [^{3}H]Leucine injected into rats 30 min prior to killing labels secretory products in endoplasmic reticulum [163]. When vesicles were treated with *C. perfringens* phospholipase C, secretory products (molecular weight 50 000–100 000) were not released [162]. Higgins and Dawson [162] have estimated that the composition of the outer leaflet phospholipids is 84% phosphatidylcholines, 8% phosphatidylethanolamines, 9% sphingomyelins and 4% phosphatidylserines and that of the inside leaflet is 28% phosphatidylcholines, 37% phosphatidylethanolamines, 6% phosphatidylserines and 5% sphingomyelins. Phosphatidylinositols could not be located directly, phospholipase C from *C. perfringens* being inactive on these lipids as is also a phospholipase C specific for phosphatidylinositols. The latter enzyme although active either on extracted lipids or detergent dispersions of microsomes, is not able to degrade phosphatidylinositols in either intact or disrupted microsomes. Higgins and Dawson [162] also found that neither phospholipase A_2 from *N. naja* nor even phospholipase C from *B. cereus* is suitable to study lipid sidedness, since they cause damage to the vesicles, resulting in leakage, access of the enzyme to the inner side and complete degradation of phospholipids except for sphingomyelins which are not attacked by phospholipase A_2. Recently, van den Besselaar et al. [18], using a purified *N. naja* phospholipase A_2, observed the hydrolysis of 55–60% of microsomal phosphatidylcholines at 0°C. At 37°C, about 70–80% of the phospholipid were hydrolyzed. The residual phosphatidylcholines, which were found to have a relatively low content of arachidonic acid, were exchangeable at 37°C by using a purified exchange protein (see below). Discrepancies in results clearly indicate that caution must be exercised when applying enzyme degradation techniques. Lysophospholipids formed by the action of phospholipase A_2 tend to disrupt the membrane structure. For example, with viruses [164,165], bacteria (*E. coli* [166] and *B. subtilis* [167]) or intracellular organelles [162], lysis has been reported. Bevers et al. [168] have confirmed that in *M. laidlawii*, an intrinsic lysophospholipase degrades the lysophospholipids formed and thus prevents lysis. However, despite the controversy over the distribution of microsomal lipids, the original proposal of Nilsson and Dallner [155,156] based on phospholipase A_2 degradation has been recently supported [169].

Phospholipase A_2 from *N. naja*, purified as previously reported [141], hydrolyzed 73% of the total phosphatidylcholines and 39% of the total phosphatidylethanolamines when mitoplasts from bovine heart mitochondria were used. With submitochondrial particles, 30% of the phosphatidylcholines and 63% of the phosphatidylethanolamines were hydrolyzed. It was observed that after Triton X-100 solubilization of the membrane, more than 90% of the phospholipids were degraded. The rates of hydrolysis of phosphatidylcholines and phosphatidylethanolamines were rapid but that of cardiolipins slow, so that

when mitoplasts and submitochondrial particles were assayed, after 60 min, about 20 and 60%, respectively, of the total cardiolipins were hydrolyzed [56]. Together with the use of fluorescamine complexed to cycloheptaamylose and the use of anti-cardiolipin antibodies, the following composition for the inner mitochondrial membrane was suggested: 28% phosphatidylcholines, 13% phosphatidylethanolamines and 6% cardiolipins on the cytoplasmic side and 11% phosphatidylcholines, 21% phosphatidylethanolamines and 18% cardiolipins on the matrix side [56]. This is again at variance with the data reported previously [156]. Work with rat liver mitochondria, using phospholipase A_2 from *N. naja*, led to the conclusion that phosphatidylcholines are evenly distributed between the two faces, 90% of the phosphatidylethanolamines being present on the cytoplasmic side and 70% of the cardiolipins on the matrix side [156]. The results of other authors do not agree with the even distribution of phosphatidylcholines in the mitochondrial inner membrane leaflets; about 65% of the phosphatidylcholines were attributed to the outer leaflet [67].

Results from different laboratories indicate the resistance of erythrocytes to lysis by phospholipase A_2 [132,170–173]. However, here again, lysis by a basic phospholipase A_2 [147] was reported under specific conditions as mentioned above. Recent studies on the action of lysophosphatidylcholine micelles have shown that several cell surface polypeptides from erythrocyte membranes are liberated, thus inducing a sodium-selective permeability defect which leads to colloid osmotic lysis. At the lowest lytic phospholipid concentration, selective disruption of the membrane protein fraction rather than gross structural reorganization of the membrane was suggested to be the primary lytic mechanism [174]. I will describe below morphological changes induced in erythrocyte membranes by different phospholipases. These changes at least in the case of phospholipase A_2 action will be explained on the basis of the retention of enzyme reaction products in the membrane.

ATP depletion of erythrocytes has been reported to enhance lysis [147,175]. Although in the case of intracellular organelles, it has been observed that serum albumin, which complexes the fatty acids and the lyso derivatives, prevents the lytic action of phospholipase A_2 [155,156], this does not occur with intact human erythrocytes [176]. Purified *N. naja* phospholipase A_2, though active in hydrolyzing phosphoglycerides, does not elicit cell hemolysis in the absence of albumin. Addition of bovine serum albumin to the incubation medium mixture brought about hemolysis. The removal of lysophospholipids was judged to be the cause of cell lysis and EDTA treatment, which chelates Ca^{2+}, led to an optimal degree of hemolysis [176]. Membrane phospholipids of ATP-depleted chicken, rat and toad erythrocytes were found to be more susceptible to phospholipase C and A_2 hydrolysis or a combination of both with sphingomyelinase [177]. Similarly, dry ether extraction of lipids of these red blood cells was enhanced by ATP depletion. In these studies [177] it was observed that trinitrobenzenesulfonate labeled 20% of the phosphatidylethanolamines of fresh chicken erythrocytes whereas 40% of these lipids were labeled in ATP-depleted cells. In fresh rat, chicken and toad erythrocytes, intramembranous particles are evenly distributed but are specifically clustered in ATP-depleted erythrocytes. In fresh chicken erythrocytes, the distance between these protoplasmic fracture face particles is 13 nm as compared to 30 nm in ATP-depleted erythrocytes. ATP depletion was found to be accompanied by dephosphorylation of certain membrane proteins. Therefore, a correlation may exist between dephosphorylation, exposure of membrane phospholipids and clustering of particles [177]. The varied accessibility of phospholipids to the action of phospholipase A_2 was also observed in *A. laidlawii*. Accessibility of the enzyme to phosphatidylglycerols decreased to 50% in cells

transporting and fermenting glucose, as compared to resting cells [178]. In studies during which the action of snake (*Hemachatus haemachates*) venom phospholipase A on erythrocytes and resealed ghosts was compared, increased availability of phosphatidylserines and phosphatidylethanolamines to the action of the enzyme was revealed. The presence of Ca^{2+} in addition elicited hemolysis of resealed cells in isotonic media in the presence of the enzyme, while normal cells are not hemolyzed under these conditions [179].

The action of phospholipase A_2 on vesicles of lecithin mixtures led to much faster hydrolysis of the more fluid species [180]. Studies on *M. laidlawii* led to the same observation, i.e., the phospholipase discriminated between phosphatidylglycerols in the liquid-crystalline state and those in the crystalline state [181].

Other authors have used phospholipase C in assessing the distribution of lipids in membranes. The action of this enzyme on erythrocytes and ghosts has found numerous applications [145,182–192]. Dense spots, presumably discrete pools of diglycerides formed during enzyme digestion, have been observed [182]. However, red cells retained their trilamellar appearance on electron micrographs. Treatment of rat and human erythrocyte ghosts with phospholipase C from *C. perfringens* hydrolyzed individual phospholipids at different rates. The more ionic lipids (phosphatidylserines, phosphatidylinositols) were little affected [183]. Phase-contrast microscopy and electron microscopy showed dense droplets associated with the membrane which were identified as diglycerides and ceramides formed by phospholipase action. Significant shrinkage of the ghosts was observed [183]. The action of sphingomyelinase on erythrocyte membranes promoted the formation of 60-Å particles which were accompanied by complementary pits in the opposite monolayer of erythrocyte membrane when studied using freeze-fracture techniques. Presumably, inverted micelles of ceramides were formed in the membrane [187]. A combination of phospholipase C and sphingomyelinase has been observed to cause lysis [187, 188]. Other studies have revealed that part of the phospholipids is available for lysis only after disruption of lipid-protein interactions by ATP depletion [175,177,189]. Degradation of erythrocyte resealed ghosts by phospholipase C has been observed to be enhanced after hypotonic lysis is used during their preparations [190,191]. Similar results on the action of a phospholipase A are reported [179].

Morphological changes consisting of the induction of invagination in erythrocytes as a result of the action of a bacterial phospholipase C have been observed [192]. In a more systematic recent investigation, morphological changes of human erythrocytes as a result of an asymmetric manipulation using phospholipases were examined [193]. On treatment with *N. naja* or bee venom phospholipase A_2, crenation of cells paralleling phosphatidylcholine hydrolysis was observed. The biconcave disc or cup-shaped form was recovered by further treatment with lysophospholipase. In contrast, phospholipase C from *C. perfringens* or *Pseudomonas aureofaciens*, or the use of fungal phospholipase D (from *Streptomyces chromofuscus*), induced invagination in cell membranes which also paralleled phosphatidylcholine hydrolysis. This morphological change was counteracted by phospholipase A_2 [193]. It could be calculated that hydrolysis of only a small amount of phospholipids would induce morphological changes. Thus, hydrolysis of about 4.6% of the phospholipids located in the outer leaflet, by phospholipase A_2 induced crenation, and hydrolysis of only 3% of the phospholipids of the outer leaflet, by phospholipase C, led to invagination. In these experiments, all phospholipases acted only on the outer leaflet, since phosphatidylethanolamines underwent little hydrolysis and phosphatidylserines were not degraded at all. Furthermore, no change in the membrane protein pattern was observed using SDS-polyacrylamide gel electrophoresis. In addition, practically no hemol-

ysis occurred. Therefore, morphological changes were correlated with the hydrolysis of the outer leaflet phospholipids. Phospholipases that do not attack human erythrocyte membranes [145], such as *Crotalus adamanteus* venom phospholipase A_2, porcine pancreas phospholipase A_2, *B. cereus* phospholipase C or cabbage phospholipase D, did not induce morphological changes. The explanation furnished by Fujii and Tamura [193] for the changes induced when effective enzymes are used is based on that given by Sheetz and coworkers [7,194,195] for crenator and cup-forming drugs. Deuticke [196] reported that hydrophobic ions induced characteristic shape changes of distinct types. Cationic drugs were suggested to intercalate mainly into the inner leaflet, because of the location of acidic phospholipid (phosphatidylserines) in this leaflet, to expand the leaflet and to induce invagination. Anionic drugs are suggested to accumulate mainly in the outer leaflet and to expand this leaflet because of the repulsion exerted by phosphatidylserines of the inner leaflet, thus inducing crenation of the cells. Asymmetric removal of all (phospholipase C) or a part (phospholipase D) of the polar head groups from the outer leaflet was suggested to shrink this leaflet, whereas the replacement of one molecule of phosphatidylcholine by one molecule each of lysophosphatidylcholine and fatty acid might expand the outer leaflet [193]. This hypothesis is supported by the fact that both the products of the action of phospholipase A_2, i.e., lysophospholipids and fatty acids, remain in the membrane and the crenation accompanying this situation is relieved by the action of lysophospholipase. In addition, exogenous lysophosphatidylcholines or fatty acids have been found to incorporate into the erythrocyte membrane, probably into the outer leaflet, and to induce crenation [193,197]. In human erythrocytes, glycocholate at low concentrations induces crenation and at high concentrations invagination [198]. It was suggested that at low concentrations glycocholate is incorporated into and expands the outer leaflet, whereas at high concentrations, its effect is to remove phospholipids and hence shrink the leaflet. An alternative explanation for the induction of invagiation by the action of phospholipase C takes into account the transmembrane movement of the diacylglycerides formed and their subsequent conversion to phosphatidic acid in the inner leaflet which results in expansion of this leaflet [199]. *C. perfringens* phospholipase C has been used in studies with rat liver membrane [200], squid axon [201], rod outer segment [202,203] and sarcoplasmic reticulum [204]. In various cases, significant quantities of phospholipids were hydrolyzed without change in the apparent organization of membranes as revealed by electron microscopy. In sarcoplasmic reticulum vesicles, 60–70% of the phospholipids, including 90% of the membrane phosphatidylcholines, were hydrolyzed [204]. Electron micrographs have provided evidence for the separation of diglyceride droplets which remain associated with the membrane [205]. Recent studies on rod outer segment have shown that 90% of the phospholipids can be hydrolyzed. Electron-microscopic monitoring continued to show the classical 'trilamellar' features of the membrane, despite the fact that only seven molecules of phospholipid per rhodopsin molecule were retained. It has been demonstrated that diglycerides formed by the action of phospholipase C begin to separate as lipid droplets once 20% of the phospholipids have been hydrolyzed. Furthermore, hydrolysis has been shown to cause the 'lateral aggregation' of rhodopsin molecules in the membrane and to perturb reversibly the properties of the photoreceptor [202].

The distribution of lipids in bacteria has been examined by using phospholipases. In gram-negative bacteria, phosphatidylethanolamines, which are major lipid constituents, are not accessible to these enzymes [206,207]. Phospholipase C from *B. cereus*, which has been shown [10] to act on spheroplasts of gram-positive bacteria without causing

lysis, was used to study different species of Bacillus. Accordingly, Bishop et al. [75] treated intact spheroplasts of *B. subtilis* and membranes derived therefrom, with phospholipase C from *B. cereus* [75]. After incubation of protoplasts for 2 h at 37°C, 45–50% of the total phospholipids were hydrolyzed which included 90% of the phosphatidylethanolamines and 70–80% of the lysophosphatidylglycerols. No lysis of protoplasts could be detected after this treatment. With isolated membranes, complete degradation of these lipids occurred and hydrolysis proceeded at a greater rate. Significantly, in these experiments, the dependence of hydrolysis on the temperature was ascribed to phase separation reducing the accessibility of the enzyme to the substrate. Control experiments with trinitrobenzenesulfonate revealed that 60% of the aminophospholipids were labeled with spheroplasts and 70% with membranes. The failure to achieve complete labeling was attributed to the bulkiness of the trinitrobenzene moiety. It was concluded that at least 60% of the phosphatidylethanolamines are located on the outer side of the protoplasts and the possibility of transbilayer movement, at least for aminophospholipids, was envisaged. However, phospholipase C did not promote lysis of the protoplasts [75]. Phospholipase C from *B. cereus* has also been used by Paton et al. [76] in conjunction with phospholipase A_2 (from *Crotalus*) and trinitrobenzenesulfonate to study the distribution of phospholipids in *Bacillus amyloliquefaciens*. Both phospholipases hydrolyzed about 70% of the total lipids of protoplasts. In this process, 90% of the phosphatidylethanolamines, 90% of the phosphatidylglycerols and 30% of the cardiolipins were degraded. However, protoplasts remained sealed as judged by the fact that the release of intracellular ribonuclease inhibitor (molecular weight 12 000) or a decrease in A_{600} did not occur during incubation. When cold-shock-treated spheroplasts were examined, hydrolysis of phosphatidylethanolamines and phosphatidylglycerols proceeded to completion and up to 80% of the cardiolipins were hydrolyzed. In intact cells, 92% of the phosphatidylethanolamines were labeled with trinitrobenzenesulfonate under conditions in which the reagent could not penetrate the membrane. These results led to the conclusion that the distribution of phospholipids is highly uneven, 70% of the total lipids being localized on the outer leaflet and the distribution of phosphatidylethanolamines being very unequal. As judged from the results of enzyme digestion, the distribution of phosphatidylglycerols and cardiolipins is also unequal, although, due to the nature of these lipids, their distribution could not be confirmed with the chemical reagent used. It should be noted that with extracted lipids, only 90% of the cardiolipins were digested by phospholipase C. The reason for the lack of complete degradation is not clear. In this microorganism, proteins constitute 60% by weight of the membrane. From the distribution of particles in freeze-fracture micrographs, and their diameter and density, it was calculated that 75% of the membrane protein is associated with the inner leaflet and this was correlated with the inequality of the amount of total lipids in the two membrane leaflets [76].

The sidedness of the lipid distribution in *B. megaterium* strain MK10 has been investigated by Demant et al. [208]. Their results do not agree with those obtained using another strain of *B. megaterium* [10]. At 37°C, 63% of the total phospholipids were removed without lysis of the membrane. More than 95% of the phosphatidylethanolamines and 3′-glucosaminylphosphatidylglycerols, but only 80% of the phosphatidylglycerols and 20% of the cardiolipins and 2′-glucosaminylphosphatidylglycerols were hydrolyzed. Isolated membranes and dispersed lipids gave similar results, consequently, it was suggested that the data reflect the specificity of the enzyme and not the distribution of lipids. Experiments carried out at 5 and 25°C, using protoplasts or isolated membranes, gave different hydrolysis patterns which led to the conclusion that 50% of the phospha-

tidylethanolamines, 70% of the 3′-glucosaminylphosphatidylglycerols and at least 40% of the phosphatidylglycerols are localized in the outer leaflet. Cardiolipins and 2′-glucosaminylphosphatidylglycerols cannot be localized since, as mentioned above, the enzyme has little effect on these substrates. The equal distribution of phosphatidylethanolamines has been confirmed by labeling with trinitrobenzenesulfonate. It was observed that the lipid phase transition in membranes and the transbilayer movement of lipids, particularly those induced by treatment with phospholipase C, do influence the rate and extent of phospholipid digestion and interfere with the interpretation of data concerning the unequal distribution of lipids. With *M. laidlawii* [168], it was similarly concluded that transbilayer movement of lipids can occur. The fact that more than 60% of the phospholipids in intact *B. megaterium* protoplasts and more than 70% in *B. subtilis* protoplasts [75] can be hydrolyzed by exogenous enzyme was interpreted as supporting this conclusion.

Recently, the distribution of lipids in the membrane of *Mycobacterium phlei* was studied using inverted vesicles of electron-transferring particles (ETP vesicles). 80% of the phosphatidylethanolamines, 24% of the phosphatidylglycerols and 13% of the phosphatidylinositols were found to be accessible to cleavage by phospholipase C from *B. cereus*. 70–75% of the phosphatidylethanolamines were found to be accessible to trinitrobenzenesulfonate or dimethylsuberimidate at 4°C. Phosphatidylethanolamines remaining after the action of phospholipase were not labeled by trinitrobenzenesulfonate, thus indicating its inaccessibility. Treatment with phospholipase C of ETP membranes from which the latent ATPase-coupling factor had been removed resulted in the hydrolysis of phospholipids to the same extent as that in ETP membranes. It was inferred that most phosphatidylethanolamines in inside-out vesicles are present in the outer leaflet and most phosphatidylglycerols and phosphatidylinositols in the inner leaflet. It was observed that degradation of 40% of the total membrane phospholipids by phospholipase C does not affect the oxidation, the coupled phosphorylation or the generation of a pH gradient, but inhibits 90–95% of the active transport of Ca^{2+} into the membrane vesicles. Treatment with phospholipase C did not render the membranes susceptible to lysis and they did not become leaky, since these membranes can maintain a proton gradient, as demonstrated by the active uptake of $^{14}CH_3NH_2$. These vesicles also accumulate amino acids against a concentration gradient.

Both *C. perfringens* and *B. cereus* phospholipases C have been applied to the study of the unequal distribution of lipids in influenza virus envelope leaflets [209–211]. The predominance of phosphatidylcholines on the outer leaflet and that of phosphatidylethanolamines and phosphatidylserines on the inner leaflet were observed and the *C. perfringens* enzyme hydrolyzed 51% of the sphingomyelins in the intact virus. Bromelain-treated, spikeless viruses [212] lost 71% of their sphingomyelins [209]. Both enzymes hydrolyzed 42–43% of the phosphatidylethanolamines and 80–81% of the phosphatidylcholines. The *B. cereus* enzyme hydrolyzed 37% of the phosphatidylserines + phosphatidylinositols. It was concluded that phosphatidylcholines and sphingomyelins are preferentially located on the outer surface where they are readily accessible to enzymes. It has been suggested that more sphingomyelins are located on the outer surface but that they oppose resistance to the *C. perfringens* enzyme [209]. In exchange studies, cholesterol was found to be equally distributed on both membrane leaflets [210]. The combination of the action of phospholipase C from both sources and the action of exchange proteins from both calf liver and bovine heart led to conflicting results [211]. It was concluded that only 30% of the total phospholipids are accessible. Phosphatidylcholines and phospha-

tidylinositols were proposed to be enriched on the outer surface, sphingomyelins enriched on the inner surface, and phosphatidylethanolamines and phosphatidylserines present in similar proportions on each surface. The reason for these discrepancies with previous results [209] is not clear. However, the authors conclude from their more recent experiments that 'the close agreement between results obtained with exchange proteins and phospholipases C demonstrates that the hydrolytic action of the enzyme does not alter phospholipid asymmetry' [211].

The use of phospholipase D in studies of lipid idstribution has been rather limited because of the lack of readily available pure enzyme [23], samples of which may alter membranes due to the presence of contaminants [213,214]. This enzyme was put to use in the study of dimyristoyl phosphatidylcholine vesicles. Its action perturbs the nature of the lipid in the outer leaflet, converting it to phosphatidic acid. Using Savoy cabbage phospholipase D, it was observed that the phosphatidic acid formed was translocated to the inner leaflet and that the rate of flip-flop of phosphatidylcholine increased from several days to 30 min [22]. Recently, a fungal phospholipase D was used in the study of morphological changes induced in erythrocytes by the action of phospholipases [193].

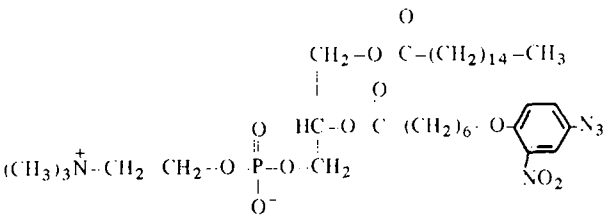
I have mentioned the existence of a lysophospholipase in the membrane of *A. laidlawii* which degrades the lysophosphatidylglycerols formed by phospholipase A_2 action [168]. Lysophospholipase has been used in studies with simple bilayer vesicles containing lysophosphatidylcholine [187,215]. A natural membrane, that of bovine chromaffin granules, is relatively rich in lysophosphatidylcholines which constitute 17 mol% of the total lipid phosphorus of this membrane [216]. Purified bovine liver lysophospholipase was found to be a convenient tool for examining the distribution and movement of this lipid in the membrane of granules [187,215]. Practically all the isolated lysophosphatidylcholines of chromaffin granules were found to be susceptible to hydrolysis by this enzyme (in fact, 94% were hydrolyzed, but 1′-alkenyl glycerophosphorylcholines of the granule membrane which constitute 9% of the lyso derivatives are not susceptible to hydrolysis). However, only 10% of the total lysophosphatidylcholines were available for hydrolysis in intact granules and with ghosts 60% were deacylated. Treatment of granules or their ghosts with trypsin did not alter the susceptibility to hydrolysis of the lipids. Of note was the fact that about 20% of the lyso derivatives were not accessible to attack from either the outside or inside, presumably because of interaction with membrane proteins [216].

VII. Exchange proteins

These proteins exchange lipids between biological membranes and artificial lipid vesicles, between serum lipoprotein complexes and membranes or vesicles and between artificial lipid vesicles [217–232]. Two types of these proteins have been reported, those which exchange specifically a given type of phospholipid and those which have a 'universal' function. As summarized previously [2], these proteins differ in their properties, especially in their isoelectric points and molecular weights. The specific protein for the exchange of phosphatidylcholines purified from bovine liver has a molecular weight of about 22 000 [218,219]. The two exchange proteins for phosphatidylinositols from bovine brain have molecular weights of 29 000 and 30 000. Two bands of exchange activity have been observed using SDS-polyacrylamide gel electrophoresis of bovine heart preparations; these activities correspond to molecular weights of 21 000 and 25 900 [221] *. A universal exchange protein from rat liver has a molecular weight of 12 000 using

* Recently, a phospholipid transfer protein specific for phosphatidylglycerols was identified in rat lung [414].

SDS-polyacrylamide gel electrophoresis and 13 500 using gel filtration [230]. Another universal exchange protein from rat hepatoma has a molecular weight of 11 200, as determined using gel electrophoresis, and 11 168 on the basis of amino acid composition [231]. Specific exchange proteins recognize the polar head group of phospholipids, interact strongly with their hydrocarbon chains and form 1 : 1 complexes [229]. Universal exchange proteins can exchange phosphatidylethanolamines, phosphatidylcholines, phosphatidylinositols and sphingomyelins, one also exchanges cholesterol [230]. The interaction of the lipid chains with a hydrophobic segment of the exchange protein has been demonstrated recently using photoaffinity techniques. Chakrabarti and Khorana [233] and Gupta et al. [234] have foreseen the potential use of photoaffinity labeling in the study of lipid-protein interactions and have synthesized different photoactive phospholipid components. Other groups have used this labeling technique [235–237]. For the study of the site of interaction between lipid chains and the exchange protein from bovine liver, 1-acyl-2-{7-(4-azido-2-nitrophenoxy)-[1-^{14}C]heptanoyl}-*sn*-glycero-3-phosphorylcholine was recently synthesized by Moonen et al. [238]. The label was incor-



porated into the protein by incubation with vesicles of this phosphatidylcholine. After activation at 340 nm and separation of the complex by chromatography, 30% of the endogenous radioactivity was linked to the protein and proteolysis showed that the 2-acyl chain of the phospholipid is bound to the peptide segment, Gly-Ser-Lys-Val-Phe-Met-Tyr-Tyr. This segment is part of a protease peptide of 65 residues bearing the cluster of hydrophobic residues, -Val-Phe-Met-Tyr-Tyr-Phe-, which may be the binding site. This segment, forming residues 26–31, exhibits an average hydrophobicity of 2440 cal per residue. It may have a β-sheet structure. Residues 21–24 and 33–36 on both sides of the hypothetical binding site have the β-turn conformation [239–242]. Work is in progress to study other aspects of the interaction of the binding site with lipid chains of phospholipids (van Deenen, L.L.M., personal communication).

An important assumption made in using exchange proteins to study lipid distribution in membranes is that only the outer leaflet components can be exchanged [229,243–248] (see, however, below) *. Consequently, Barsukov et al. [248] have used exchanged

* A number of artifices have been used to separate vesicles. For example, Forssman antigen was included in one population of vesicles which could then react with the antibodies [249]. Others introduced phosphatidic acids into vesicles which they could separate after the exchange reaction using DEAE-cellulose chromatography [250,251]. In other experiments, one type of vesicle contained in addition to lipids (cholesterol/egg lecithins), *N*-palmitoyl-DL-dihydrolactocerebroside, which can be retained by *Ricinus communis* lectin and thus allow the separation from vesicles of the lipid mixture which does not contain a ligand for the lectin [252]. Yet another way of separating vesicles is to use 'heavy molecules' for the preparation of one type of vesicle (dense vesicles). These molecules were synthesized by bromination of dioleoyl phosphatidylcholine and the exchange examined between single bilayer vesicles obtained from the brominated lipid and dioleoyl phosphatidylcholine [253]. In the latter example, only the molecules from the external leaflet were exchanged and flip-flop of lipid was so slow that it could not be measured [253]. Another fact of interest is that the transbilayer movement of phosphatidylethanolamines in mixed phosphatidylcholine and phosphatidylethanolamine single-walled vesicles was measured simply by the use of amino group reagents; a half-time of at least 80 days at 22°C was estimated [254].

proteins as a tool in the study of the sidedness of lipid distribution. A fraction of exchange protein from rat liver was employed for the examination of spheroplast membranes of *Micrococcus lysodeikticus*, of which the lipids contain only phosphatidylglycerols, cardiolipins and phosphatidylinositols. Approximately half of the phosphatidylglycerols and diphosphatidylglycerols was transferred by this protein to phosphatidylcholine liposomes. Phosphatidylinositols were not transferred. Ghosts of *M. lysodeikticus* and pronase-treated spheroplasts were examined concurrently. Pronase treatment removed the shielding of an additional part of the external lipids, so that 80% of the total phosphatidylglycerols and 20% of the phosphatidylinositols were transferred together with half of the cardiolipins. It was concluded from these results that cardiolipins are evenly distributed, and that phosphatidylglycerols are located predominantly in the outer and phosphatidylinositols in the inner leaflet. Control experiments were performed using phospholipase A_2 from *Naja naja oxiana*; the same results were obtained with regard to phosphatidylglycerols and cardiolipins. Other controls were carried out using phospholipase C which confirmed the results on phosphatidylinositols obtained either with spheroplasts or with pronase-treated spheroplasts. 70% of the phosphatidylglycerols were hydrolyzed with native spheroplasts; this was taken to indicate the difference in accessibility between phospholipase C and either phospholipase A_2 or transfer protein. The eventual leakiness of spheroplasts was controlled by monitoring the E_{260} value (for nucleotidic material) of their supernatant. Lenard and Rothman [210] and Rothman and coworkers [211] also used, independently, two phospholipid exchange proteins in conjunction with two phospholipases to study the transbilayer distribution of phospholipids in the influenza virus envelope and found that 30% of the total phospholipids were accessible and 70% inaccessible. Consequently, there is an unequal distribution of lipids and, as stated above, the outer leaflet was found to be enriched with phosphatidylcholines and phosphatidylinositols and the inner leaflet with sphingomyelins. Phosphatidylethanolamines and phosphatidylserines appear to have an equal distribution in the two leaflets [210,211]. The action of phospholipase C in these studies [210,211] led to results similar to those obtained with exchange proteins. Therefore, it was concluded that the hydrolytic action of these enzymes does not alter the phospholipid asymmetry. A rate of transbilayer movement of at least 30 days for sphingomyelins and of 10 days for phosphatidylcholines at 37°C was estimated by using bovine heart exchange protein which is supposed to be non-perturbing.

Investigations on the phosphatidylcholine distribution in intact human erythrocytes using exchange proteins have confirmed that 75% of these lipids are exchanged [255]. In rat erythrocytes, however, the exchange amounted to 50–60% of the phosphatidylcholines, though after the rapid exchange, the residual phosphatidylcholines continued to be exchanged slowly [255–257]. The extent to which phospholipids of erythrocytes and resealed ghosts are exchanged has been observed to differ [257]. When purified bovine heart phospholipid exchange protein was employed, the transfer of phosphatidylcholines (and to a lesser degree that of sphingomyelins, phosphatidylinositols and lysophosphatidylcholines) between phospholipid/cholesterol liposomes and labeled sealed ghosts was observed; 75% of the phosphatidylcholines of sealed ghosts were readily available to exchange, the remaining 25% being exchanged more slowly. However, the presence of exchange protein did not accelerate the exchange of phospholipids between intact red blood cells and liposomes. With inside-out erythrocyte membrane vesicles, 37% of the phosphatidylcholines were found to be readily exchanged, the remainder doing so at a slower rate. It was concluded that phosphatidylcholines are asymmetrically distributed

and that the asymmetry is mostly maintained during the formation of inside-out vesicles though being partially altered during ghost preparation. These observations are in good agreement with those of other authors on the failure of exchange of erythrocyte phosphatidylcholines to occur when bovine liver phosphatidylcholine exchange protein is used [243,250]. A half-time of equilibration of 2.3 h was estimated for resealed ghosts and of 5.3 h for inside-out vesicles. To explain these observations the plausibility of a correlation between changes in the rate of lipid movement and 'alterations in the membrane structure' was discussed by Bloj and Zilversmit [257].

Bovine liver exchange protein has been used to study the unequal distribution of phosphatidylcholines in murine LM cell plasma membranes. Cells were grown in the presence of radioactively labeled phospholipid precursors, then latex phagosomes were prepared and the exchange of phosphatidylcholines with either unilamellar vesicles or unlabeled phagosomes followed. About 52% of the phosphatidylcholines were readily exchanged; a second pool exchanged more slowly [69]. In the same study, 90 and 24% of phosphatidylethanolamines were found to be labeled by trinitrobenzenesulfonate when phagosomes and phagosomes prepared from prelabeled cells, respectively, were examined. Sphingomyelins were located, from indirect evidence, on the outer leaflet. No major difference was found in the distribution of acyl chains within a given phospholipid class but differences in the acyl chain composition among different lipid classes resulted in an enrichment of unsaturated fatty acyl chains on the cytoplasmic face [69]. These results are not in good agreement with others obtained using the same cells [70]. Another more subtle discrepancy is that in the latter studies [70], the acyl chains of phosphatidylethanolamines from the outer leaflet appeared more saturated, whilst the former study [69] does not indicate such a difference for any particular lipid class.

Based on the exchange of the outer leaflet lipids of biological membranes as a result of the use of exchange proteins, different experiments were carried out in order to manipulate membrane lipids *. Rat liver exchange protein incorporated phosphatidylcholines into spheroplasts prepared from *M. lysodeikticus*. About half of the bacterial total phospholipids was thus replaced by phosphatidylcholines, a lipid class which bacteria do not synthesize [263]. As a result of this exchange, it was observed that endogenous respiration of spheroplasts was not influenced but that the modified spheroplasts were found to be osmotically more stable. The permeability of these spheroplasts to low molecular weight molecules decreased as well as the rate of exogenous substrate (NADH, malate) oxidation and ferricyanide reductase activity. After osmotic lysis, the enzyme activities were restored [263]. These experiments are of particular value, since they constitute an alternative means of manipulating phospholipids. For intact protoplasts, exchange concerned essentially the outer leaflet, since 90% of the phosphatidylcholines incorporated were susceptible to hydrolysis by phospholipase A_2. Under the same conditions, less than 50% of the normal protoplast phospholipids, mainly phosphatidylglycerols and diphosphatidylglycerols, are hydrolyzed [263]. As discussed below, this property of exchange was also used in the analysis of microsomal and mitochondrial membranes. However, as for bacteria [13,14], rapid transbilayer movement was observed for some intracellular membranes. Thus, Zilversmit and Hughes [17] used rat liver microsomes and the exchange of the in vivo labeled phospholipids was studied. The half-time of transbilayer movement was found to be of the order of minutes. Using an excess of mitochondria and

* For some other methods used to manipulate membrane lipids see Refs. 258–262.

phospholipid exchange proteins derived from bovine heart and bovine and rat liver, labeled phosphatidylcholines, phosphatidylethanolamines, phosphatidylserines and phosphatidylinositols were found to act as a single pool and were exchanged to an extent of 85–95% in 1–2 h [17]. The high latency of mannose-6-phosphate phosphohydrolase activity and the impermeability of microsomes to EDTA showed that lysis did not occur. It was concluded [17] that if microsomal membranes are largely composed of phospholipid bilayers, one or more of the phospholipid classes undergoes rapid translocation between the inner and outer membranes.

Van den Besselaar et al. [18] have used the purified phosphatidylcholine specific exchange protein from bovine liver to examine exchange of rat liver microsomal phosphatidylcholines with egg phosphatidylcholine dispersions. Exchange reached completion at 25 and 37°C. When lipids were extracted from the microsomes and vesicles prepared no more than 60% of the phosphatidylcholines were exchanged. At 8 and 0°C, the exchange kinetics are complex. Exchange of phosphatidylcholines does not affect the permeability of microsomes to mannose 6-phosphate or that of phosphatidylcholine vesicles to Nd^{3+}. Parallel studies on mitochondrial inner membranes showed the complete exchange of phosphatidylcholines, whilst with vesicles prepared from extracted lipids, only 65% were exchanged. Here again, at 8 and 0°C, exchange is not effected, as if a single pool were available at 37°C. A rapid equilibrium between different pools was suggested to take place. However, the nature and location of the pools remain to be established. In other studies with rat liver microsomal membranes, it was found that a part (20%) of the phosphatidylinositols was not accessible for exchange when phospholipid exchange protein from bovine brain was used [264]. The above-mentioned observation seems to indicate that for some intracellular active membranes, a high rate of flip-flop may be expected as is revealed also by NMR studies (see below). However, this did not appear to be the case with phagosomal membranes [265]. On the other hand, experiments with membranes of different origin, including microsomal and mitochondrial (see below) membranes, were based on the assumption of the exchange of lipids from only one leaflet with no appreciable transbilayer movement. Therefore, the 'in situ' manipulation of lipids was attempted. Different examples of this type of manipulation have been reported. Lysophosphatidylcholines were introduced into intact rat liver microsomes by using exchange proteins. Cytochrome *P*-450 was converted into the inactive cytochrome *P*-420. When these microsomes were incubated with phosphatidylcholines in the presence of lipid exchange proteins, lysophosphatidylcholines were partly replaced by phosphatidylcholines and the inactive cytochrome *P*-420 was reactivated. Reactivation with phosphatidylethanolamines failed to occur, indicating the specificity of the phospholipid needed for reactivation [266]. Interesting observations have been made on the activity of microsomal glucose-6-phosphatase [169]. Nilsson et al. [267] have shown that this enzyme is constituted of two parts, a specific carrier protein mediating the transport of the otherwise impermeant glucose 6-phosphate across the membrane and a phosphatase localized at the luminal surface [268,269]. Treatment of intact microsomes with proteases or diazosulfanilate does not inactivate the catalytic site as evidenced by its activity in detergent-dispersed microsomes and, since the permeability of microsomes for mannose 6-phosphate, nucleosidediphosphatase or dextran (molecular weight 70000) does not change after modification with protease or diazosulfanilate, a component of the glucose 6-phosphate system exposed to the external surface and involved in its transport is thought to be involved in the process and to be attacked by the reagents [267]. Supplementation of intact rat liver and hepatoma microsomes with exogenous aminophospholipids by using exchange pro-

teins was observed to prevent the decrease in glucose-6-phosphatase activity during the incubation, whereas the introduction of exogenous phosphatidylcholines had no protective effect. With deoxycholate-dispersed hepatoma microsomes, however, introduction of additional phosphatidylcholines causes activation, whilst phosphatidylethanolamines have little effect [169]. It was concluded that the activity of the transport unit depends on phosphatidylethanolamines and phosphatidylserines and that of the catalytic unit on phosphatidylcholines. It was suggested that the diminished glucose-6-phosphatase activity in hepatoma microsomes may be partly attributed to a low level of phosphatidylcholines. In deoxycholate-disrupted liver microsomes in which the phosphatidylcholine content is much higher, the glucose-6-phosphatase activity is also much higher and exogenous phosphatidylcholine does not affect the enzyme activity. From this it was concluded that the two components of glucose-6-phosphatase are surrounded by two different lipid environments. In other experiments, results obtained using hepatoma mitochondria led to the conclusion that only exposed phosphatidylcholines are readily exchanged [270]. Introduction of phosphatidylcholines into hepatoma mitochondria by the use of phospholipid exchange protein resulted in a considerable activation of the monoamine oxidase activity, an outer mitochondrial membrane enzyme of low activity in hepatoma mitochondria [271,272]. It is noteworthy that, whatever the cause of the discrepancies between the results of workers who maintain the principle that exchange affects only the external leaflet and those who interpret their results as being indicative of rapid transbilayer movement, methods of manipulating membranes using exchange protein seem applicable. However, the possibility of transbilayer movement should be considered very seriously, since not only experiments with some organelles indicate that this occurs [17,18] but also NMR results (see below) and studies with lipid vesicles support this possibility.

[*choline-methyl*-^{13}C]Phosphatidylcholines were introduced into the outer monolayer of phosphatidylcholine vesicles with bovine liver exchange protein. When, at 30°C, dioleoyl [*choline-methyl*-^{13}C]phosphatidylcholine or dimyristoyl [*choline-methyl*-^{13}C]-phosphatidylcholine was introduced into the corresponding non-labeled liposomes, and the rate of flip-flop was estimated by using ^{13}C-NMR, half-times of the order of several days were observed. However, when dioleoyl [*choline-methyl*-^{13}C]phosphatidylcholine was introduced into vesicles of dimyristoyl phosphatidylcholine, it could 'migrate' from the outer to the inner leaflet with a half-time of less than 12 h. Differential changes in lateral packing of the two monolayers were suggested to constitute the 'driving force' [273], the surface area of dioleoyl phosphatidylcholine and dimyristoyl phosphatidylcholine being, respectively, 78 and 60 Å^2 [274]. Similarly, lysis of molecules from the outer leaflet of dimyristoyl phosphatidylcholine vesicles by phospholipase D, which leads to the formation of phosphatidic acid in this leaflet, resulted in a half-time of the transbilayer movement of 30 min [22].

An exchange protein has been reported to enhance the exchange of cholesterol as well as that of other lipids [230]. In addition, the possibility of the non-catalyzed transbilayer movement of cholesterol has been examined. Certain observations have indicated such a possibility and a rapid rate in erythrocytes [275–278] and vesicular stomatitis virus [279], others a slow rate in the influenza virus membrane [210] and in *Mycoplasma galliseptum* [280]. Even with small vesicles, contradictory results have been reported. Some authors measured a very slow rate [281,282] whereas others detected rapid movement [252,283].

The presence of proteins in artificial membranes has not led to a general concept of the enhancement of transbilayer movement by these molecules. Thus, studies with soni-

cated glycophorin containing dioleoyl [*choline-methyl*-^{13}C]phosphatidylcholine vesicles (in which the glycophorin shows the natural orientation) indicate that the distribution of lipids is comparable to that of protein free vesicles. The distribution of palmitoyl lysophosphatidylcholine in these vesicles is unequal, the inner leaflet being richer, when compared to vesicles of the mixture in the absence of glycophorin. It has been observed that lysophosphatidylcholine, added to the pre-existing glycophorin-containing dioleoyl phosphatidylcholine vesicles and incorporated into the outer leaflet, moves to the inner leaflet with a half-time of 1.5 h at 4°C. Moreover, if cosonicated vesicles (containing both lipids and glycophorin) are submitted to the action of lysophospholipase, complete degradation of the lyso compound takes place. A half-time of 1 h at 37°C has been measured for the movement of lysophosphatidylcholine [284]. Other experiments have shown that the increase in the rate of transbilayer movement of dioleoyl phosphatidylcholine in sonicated simple vesicles in the presence of glycophorin is several orders of magnitude greater. When ^{13}C-labeled molecules were introduced by means of phospholipid exchange protein from glycophorin-containing donor vesicles to non-labeled acceptor vesicles, it was estimated that after 3.5 h of incubation only 5% of exchange labeled molecules exist in the inner leaflet. Conversely, if glycophorin-containing vesicles were acceptor vesicles then 25% of the labeled molecules were retained after 2 h of incorporation into the inner layer (normally one would expect 33% of the label in the inner leaflet; however, the possibility cannot be excluded that some 'glycophorin-containing vesicles' are poor in glycophorin. The average number of molecules of this protein per vesicle is four to five [285]).

Recent investigations [286] on large unilamellar vesicles of phosphatidylcholines prepared by ether vaporisation [287], small cholate-dialysis vesicles and cytochrome oxidase-containing vesicles, prepared both by the cholate-dialysis method and by a direct incorporation method, showed that exchange proteins can, in each case, reveal two pools; one rapidly exchanged and the other very slowly exchanged. The size of the pools depends on the nature of the vesicles so that in large vesicles, the pools are of equal importance. In small vesicles 65% of the phosphatidylcholines are exchanged and 35% only slowly exchanged, and in cytochrome oxidase-containing vesicles 70% of this lipid is exchanged and 30% slowly exchanged. It was concluded that the extremely slow rate of exchange of the second pool reflects the virtual non-existence of flip-flop in any of the three model membranes used and, in particular, the absence of flip-flop in vesicles with a phospholipid-to-cytochrome oxidase ratio of 2 : 1 is in contrast to the above-mentioned observations with glycophorin-containing vesicles.

VIII. Physical techniques

Among these techniques, the most fruitful for asymmetry studies is the NMR technique which was first used in this field by Bergelson and his colleagues [288,289]. NMR has been employed extensively for investigations on the distribution of lipids in vesicles formed of a single phospholipid or mixtures of phospholipids, with or without cholesterol [22,186,271,288–294]. Large phospholipid vesicles, with or without intrinsic proteins of erythrocyte membranes, have also been examined using NMR [295].

The unequal distribution of lipids, as revealed by ^{1}H-, ^{31}P- and ^{13}C-NMR techniques, was demonstrated mostly in artificial membranes and will not be detailed at length here (for more information see references cited in Ref. 1). Very briefly, the principle is that for a given resonant nucleus, the absorption attributable to both leaflets of a vesicle can

be estimated and, hence, the ratio of the number of the same molecules present in the outer and the inner leaflets investigated. This is possible, even if, as occurs for ^1H resonance, absorptions from molecules of both leaflets are superimposed, since the use of shift reagents displaces the resonance position of exposed molecules *. Recently, this technique was used to study the distribution of phosphatidylcholine molecules in sarcoplasmic reticulum, after in vivo enrichment of phosphatidylcholines by feeding rats with [*methyl*-^{13}C]choline [297]. The transbilayer movement of phosphatidylcholines and lysophosphatidylcholines of sarcoplasmic reticulum has also been studied [298]. The NMR technique was used to monitor the transbilayer movement of lipids when asymmetric perturbation was performed on one leaflet. Thus, as reported above, the action of phospholipase D on such bilayer vesicles of phosphatidylcholines led to relatively rapid transbilayer movement of the phosphatidic acid generated by enzyme action [22]. On the other hand, exchange protein-mediated modification of the fatty acid composition of the outer leaflet of phosphatidylcholine vesicles led to the observation that this lipid effected transbilayer movement. This study was made possible by the use of ^{13}C-NMR [273].

Another use of NMR, developed in relation to the architecture of membranes, is the examination of the nature of lipid arrangement in membranes. To appreciate this recent development, which may have important implications with regard to the distribution of lipids, a short introduction to pertinent aspects of the phase behavior of some lipid classes is appropriate (for recent summaries on lipid phase behavior, see Refs. 1 and 299).

Pioneer work by Luzzati and coworkers [300–302], using X-ray diffraction, indicated that lipids of biological membranes exist in a bilayer structure under physiological conditions, and depending on the experimental conditions, show different polymorphic arrangements. Reiss-Husson [303] has reported the coexistence of the hexagonal type II phase in egg yolk phosphatidylethanolamines with the bilayer phase between 25 and 35°C (see also Ref. 304). The possibility of the hexagonal type II phase existing has since been reported for phosphatidylethanolamines of other origin by using X-ray diffraction [305, 306] and ^{31}P-NMR [21]. The enthalpy of transition associated with the bilayer-hexagonal II transition was found to be very low [307], which is consistent with NMR [308] and ESR studies [309], indicating little change in the order parameter during this transition. Concerning the behavior of cardiolipins, X-ray studies show that between c = 0.3 and 0.53% (i.e., 30–53% lipid in aqueous medium) the structure is lamellar, between c = 0.85 and 0.95% a hexagonal type II phase exists and between c = 0.53 and 0.85% the two arrangements coexist. However, at all concentrations, Ca^{2+} precipitates the lipid. This precipitate shows the hexagonal type II arrangement [310]. Cardiolipins obtained as simple vesicles also undergo a phase change to the hexagonal type II phase under the influence of Ca^{2+} [311]. Neutralization of the charges on the head groups has been suggested to explain the property of these lipids, which in the presence of Ca^{2+}, behave as if they were dehydrated, the unlimited swelling of these lipids being attributed to electrostatic repulsion between their head groups [305]. Phosphatidylserines are 'bilayer formers'.

* Shift reagents are anions such as $Fe(CN)_6^{3-}$ or cations such as Mn^{2+}, Co^{2+}, Eu^{2+}, Pr^{3+}, Nd^{3+}, Yb^{3+} and Dy^{3+}. These ions penetrate leaky membranes and displace the resonance position of molecules of both sides. If membranes are sealed, only the resonance position of exposed molecules is displaced. Chelation of some of these ions, for example, using EDTA or chromatography, allows the removal of the shift reagent from the relevant side. Molecules of both sides can be studied by using a suitable choice of experimental conditions to exploit the different possibilities thus offered. (For an interesting review on the use of the shift reagents, see Ref. 296.)

Dilauroyl phosphatidylserine shows a lamellar structure between c = 0.3 and 0.8% [305]. In vitro experiments with lipid mixtures of the composition of the inner leaflet of erythrocyte membranes (relatively rich in phosphatidylserines) led to the observation that addition of Ca^{2+} triggers the bilayer-hexagonal type II transition [312], presumably by segregating phosphatidylserine molecules which exert a bilayer-stabilizing effect in mixtures. Studies with phosphatidylserine sonicated vesicles have shown that neutralization of head groups by Ca^{2+} leads to precipitation of aggregates termed 'cochleate cylinders' [313–315]. However, the formation of the hexagonal type II phase is reported for phosphatidic acids in the presence of Ca^{2+} [316] and for monoglucosyldiglycerides [317].

Lipid mixtures separated from biological membranes show, in general, the bilayer structure [1,300,301,318], although exceptions to this rule have recently been reported (see below). It was assumed that in mixtures of extracted lipids, those lipids tending towards the bilayer structure impose mostly this arrangement; lipids able to swell unlimitedly favor this behavior so that bilayers which are able to swell unlimitedly are formed [305]. NMR investigations have advanced the recognition of arrangements interpreted as being intermediate between the bilayer and hexagonal type II phases in artificial mixtures of lipids, extracted lipids or even in natural membranes.

The application of ^{1}H-NMR led Davis and Inesi [19] to conclude that in sarcoplasmic reticulum membrane, 20% of the lipids may experience isotropic motion. Studies on the ^{31}P-NMR spectra of known phospholipid dispersions led to the recognition of features attributed to bilayer, hexagonal type II and 'intermediary phases' such as cubic, rhombic, inverted micellar and vesicular phases for which isotropic motion is conceivable [20,21, 299,319]. Using this tool (see Fig. 1), it has been confirmed that phosphatidylcholines

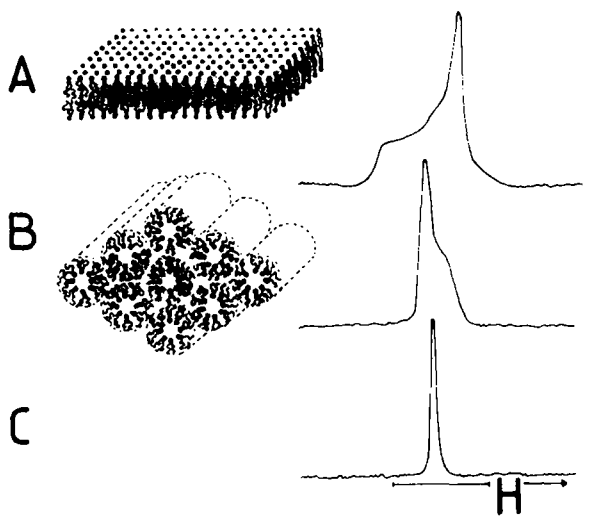


Fig. 1. Schematic representation of the sensitivity of the motion experienced by phospholipid molecules to the nature of the phase in which they participate. Different phases (left) and the corresponding ^{31}P-NMR spectra (right) are reproduced. (A) Bilayer (aqueous dispersion of egg yolk lecithin); (B) hexagonal type II phase (soya phosphatidylethanolamines), and (C) cubic, rhombic, inverted micellar (micellar, vesicular) phases (not shown) (a mixture of soya phosphatidylethanolamines (85%) and egg yolk lecithin (15%)). Spectra were obtained at 36.4 MHz, at 30°C, in the presence of broad-band proton decoupling. The bar represents 50 ppm. (Redrawn from the reports by Cullis and de Kruijff [299] and Cullis and Hope [334].)

[319–322] as well as sphingomyelins [299] in mixtures of lipids favor the bilayer arrangement and that their progressive addition to lipids showing the hexagonal type II arrangement leads to the phase transition. The formation of intermediary phases at intermediate concentrations of bilayer formers has also been observed. It was noted that both saturated and unsaturated phosphatidylcholines retain the bilayer structure in the presence of cholesterol [320]. However, in mixtures of unsaturated (but not saturated) phosphatidylcholines and phosphatidylethanolamines, in which the bilayer arrangement is elicited by the presence of the phosphatidylcholines, an equimolar concentration of cholesterol promotes the formation of the hexagonal type II phase [323]. The formation of intermediary phases (between the bilayer and hexagonal type II phases) by the addition of Ca^{2+} to cardiolipins [324] has also been reported. Phosphatidylserines promote the formation of the bilayer structure in mixtures with phosphatidylethanolamines [325]. Addition of Ca^{2+} causes rearrangement to the hexagonal type II phase either by segregation of phosphatidylserine molecules or because of an altered 'shape' of the Ca^{2+}-phosphatidylserine complex. Thus, in a model membrane system consisting of 20 mol% bovine brain phosphatidylserines and 80 mol% egg yolk phosphatidylethanolamines, addition of Ca^{2+} promotes the isothermal bilayer-to-hexagonal type II transition. Displacement of Ca^{2+} by a local anesthetic such as dibucaine reverses the arrangement to that of the bilayer [325].

Freeze-fracture studies with model systems, cardiolipins [324] and mixtures of cardiolipins and phosphatidylcholines [326,327] show that the presence of Ca^{2+} induces the formation of particles and pits which are observable on the freeze-fracture faces. The transition of cardiolipins from the bilayer to the hexagonal type II phase induced by addition of Ca^{2+} occurs through an intermediary phase formed of particles 70 Å in diameter. ^{31}P-NMR shows the isotropic motion of molecules. This phase, existing below Ca^{2+} : cardiolipin ratios of 0.6, is presumed to be formed of inverted micelles [326]. In an equimolar mixture of lecithins and cardiolipins at a low Ca^{2+} : cardiolipin ratio, particles of 100 Å and pits of 70 Å in diameter are observed. Furthermore, if Ca^{2+} is removed by a chelating agent (EGTA), the particles and pits disappear and smooth fracture faces result. The particles are suggested to represent inverted micelles sandwiched between lipid leaflets [326]. Here again, ^{31}P-NMR confirms the presence of an arrangement attributable to an intermediary phase. Similarly, the presence of such phases can be detected by the use of ^{31}P-NMR and freeze-fracture studies of different mixtures of lipids such as phosphatidylcholine/monoglucosyldiglyceride or phosphatidylcholine/phosphatidylethanolamine mixtures in the presence of cholesterol [327].

Studies on different biological membranes have resulted in spectra of typical bilayer structures either for intact membranes or liposomes of extracted lipids [294,318,328, 329]. In the case of erythrocytes, the bilayer arrangement was conserved after enzyme degradation when combinations of different lipolytic enzymes (phospholipase A_2, phospholipase C and sphingomyelinase) were used. The ^{31}P-NMR spectra of erythrocyte ghosts indicate that residual lipids, including lyso derivatives, retain the bilayer arrangement even though more than 90% of the phospholipids are degraded [330]. The ^{31}P-NMR features of bovine, rat [15] and rabbit [16] microsomal fractions led to the suggestion that a part of the lipids may experience isotropic motion, probably due to the presence of inverted micellar or short cylindrical hexagonal type II arrangements of lipid inside the bilayer [15,16]. It was suggested that membrane proteins and particularly cytochrome *P*-450 [16] allow the isotropic motion of lipids. This has been supported by reconstitution experiments [16]. The presence of a non-bilayer arrangement is tempera-

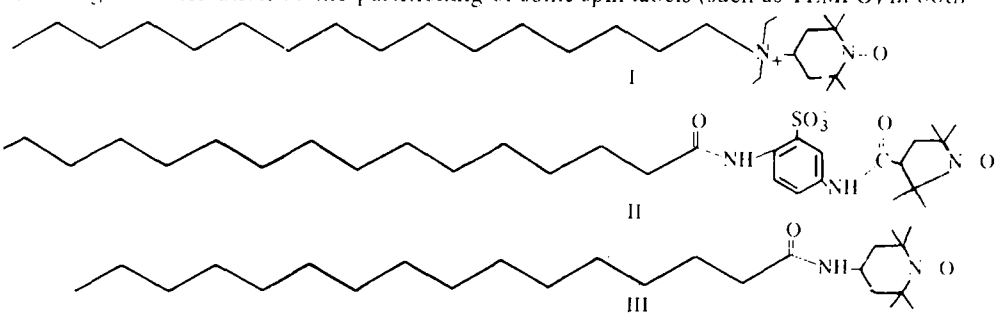
ture dependent. At 4°C, mainly the bilayer structure is observed and transbilayer movement is greatly decreased [18]. Isotropic movement of a fraction of the lipids in sarcoplasmic reticulum membrane preparations was suggested on the basis of ^{31}P-NMR [299] which confirms results previously presented on the basis of ^{1}H-NMR [19]. ^{2}H-NMR has also given indications that a part of the inner mitochondrial membrane lipids may experience isotropic motion [331]. This has been confirmed by using ^{31}P-NMR [299]. With lipid extracts from inner mitochondrial membrane [327], rod outer segment [332] and *E. coli* [333], 'lipid particles' were revealed using freeze-fracture studies and 'pits' were observed on fracture faces. ^{31}P-NMR confirms that the isotropic movement of lipids does occur. For example, lipids in rod photoreceptor membrane are in the bilayer state as assessed using ^{31}P-NMR at 37°C. However, extracted lipids are in the hexagonal type II phase and isotropic motion was detected [332]. Non-bilayer structures have been suggested [334] to be involved in processes of cell fusion promoted by some 'fusogens' [335,336]. The well known role of Ca^{2+} in promoting fusion [337–339] has also been attributed to its ability to elicit the inverted micellar structure at the fusion interface [299].

Apart from the possible involvement of the existing or induced intermediary phases in the fusion processes, the role of such phases in the transbilayer movement of lipids should be considered in connection with the genesis of membranes and as a factor in shaping the distribution of lipids [1,299]. In addition, the possible involvement of inverted micelles in the process of transport of molecules was envisaged [299].

Thus, NMR techniques have contributed greatly to the measurement of the unequal distribution of lipids in lipid vesicles, to the recognition of their transbilayer movement and to the realization that non-bilayer arrangements may exist or be induced in some biological membranes. The electron spin resonance (ESR) technique used by Kornberg and McConnell [340] has demonstrated the slowness of the flip-flop movement in dipalmitoyl phosphatidylcholine vesicles. A spin-labeled marker in which TEMPO-choline replaced choline in the phospholipid molecule was introduced into phosphatidylcholine vesicles and asymmetrically reduced by ascorbic acid. The transbilayer movement was studied and found to be a slow process with a half-time of 6.5 h at 30°C. This work initiated numerous studies on the transbilayer movement of lipids resulting in the confirmation of the slowness of this movement in vesicles. Recently, a synthetic spin label was introduced asymmetrically into a biological membrane by using exchange proteins [246]. In this experiment, 2-(5-doxylpalmitoyl)phosphatidylcholine was incorporated into the membrane of the isolated inner membrane-matrix complex of rat liver mitochondria. Rat or bovine liver exchange protein was used to stimulate the exchange. The signal thus introduced into the mitochondrial membrane was destroyed by ascorbic acid treatment at 0°C, indicating that the label was incorporated into the outer leaflet and that flip-flop did not occur. This has been confirmed by labeling the mitochondrial preparation with either TEMPO-phosphatidylcholine or 2-(5-doxylpalmitoyl)phosphatidylcholine in the absence of exchange protein. When the latter spin label is used, ascorbate reduces the ESR signal by approx. 50%. With the former, ascorbate reduces all the label. It was suggested that both leaflets contain labeled molecules introduced by fusion in the membrane, but that when the label is on the head group the mitochondrial content is able to reduce the label and to introduce 'anisotropic' distribution without the need for ascorbate [246]. This situation led to the observation of a half-life of at least 24 h at 22°C for the spin label. In a parallel experiment during which human red blood cells were assayed with the same spin labels, the reducing power of the intracellular compartment was again observed. The

inwardly exposed spin label of TEMPO-phosphatidylcholine was reduced, then the flip-flop was examined and found to be so slow that the half-life could not be measured [341]. The head group of inwardly oriented TEMPO-phosphatidylcholine was not reduced when ghosts were used, thus confirming the reducing power of the intracellular compartment. On the other hand, when TEMPO-phosphatidylcholine, in which the label is located at the head group, was used, the exchange protein did not stimulate the incorporation into mitochondrial or erythrocyte membranes, therefore confirming the specificity for the head group of the phospholipid to be exchanged. The incorporation of lipid, which is thought to occur by fusion, concerned both leaflets of the erythrocyte and mitochondrial membranes in the absence of exchange protein. Another observation was that sonicated spin-labeled phosphatidylcholine induced crenation in erythrocytes. This was attributed to the formation of lysophosphatidylcholine and free fatty acid during the sonication. When vesicles were treated with albumin, morphological changes in erythrocytes were prevented [341].

Attempts have been made to design spin labels which do not cross the membrane and hence label only one leaflet. Labels I (positively charged), II (negatively charged) and III (uncharged) were synthesized [342]. Using yeast cells, it was observed that labels I and II remained exposed on the outer surface. Comparison of the spectra of labels I and II showed that label I was more immobilized. Using line-broadening reagents such as $Fe(CN)_6^{3-}$ ($K_3(Fe(CN)_6)$) and Ni^{2+} ($NiCl_2$), which remove the signal from spin labels located in the same environment [343,344], it was observed that the spectra of labels I and II vanished when Ni^{2+} and $Fe(CN)_6^{3-}$, respectively, were used. Since these agents are non-permeant, this observation indicates that flip-flop does not occur during the experiments. When label III was assayed with yeast cells, the spectra changed only slightly on addition of broadening reagent. However, when sarcoplasmic reticulum vesicles were assayed under the same conditions, 60% of the signal of label III was removed. These observations have been interpreted in terms of the distribution of label III among the numerous intracellular membranous structures of the yeast cell and between the two leaflets of sarcoplasmic reticulum [342]. Thus, the charged nature of labels I and II which prevents flip-flop may allow examination of the change in the physical stage at or near the membrane surface. Further studies probing the depth of the bilayers, carried out by Hubbell and McConnell [345] and others [346,347], have led to the definition of the order parameter, indicating a gradient of fluidity through the acyl chains of lipids. The original observation of the partitioning of some spin labels (such as TEMPO) in both

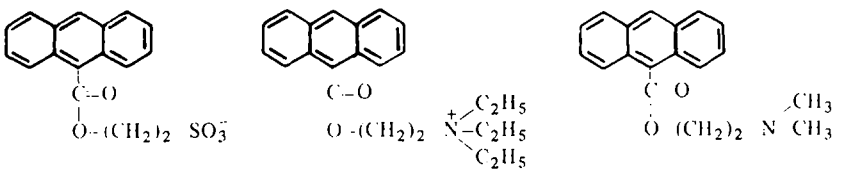


the aqueous space and hydrophobic core of membranes [348–350], and the presence of discontinuities in the Arrhenius plots of the relative fraction of spin label in the hydrocarbone core as a function of temperature gave information on membrane asymmetry. The onset and completion of the lateral phase transition, indicated by temperatures t_1 and

t_h (lower and higher, respectively), have been defined for phospholipid dispersions and bacterial (*E. coli*) [351] and mammalian cell membranes [352]. Using the hydrophobic spin label, 2-butyl-2-pentyl-4,4′-dimethyl-3-oxazolidine-*N*-oxyl (5N10), a nitroxide derivative of decane, two characteristic temperatures were found for *E. coli* inner membrane whereas four characteristic temperatures were observed for LM cells [353]; the latter temperatures are approx. 15, 21, 30 and 37°C. It was hypothesized that these four temperatures correspond to t_l and t_h of the two leaflets of the membrane, the values for one leaflet being 15 and 30°C and those for the other 21 and 37°C [352]. Since the hydrophobic probe 5N10 experienced transition in both leaflets, to test the above hypothesis a glucosamine derivative of 12-nitroxystearic acid (2-(10-carboxydecyl)-2-hexyl-4,4-dimethyl-3-oxazolidine-*N*-oxyl or 12-NS-GA), which because of the polar group cannot flip from one leaflet to another [353], was used in addition to 5N10 [354]. Bacterial and mammalian cell membranes were used and Arrhenius plots were obtained from $\log h_H/h_P$ vs. $1/k$ (h, height of signals in hydrocarbon (H) and polar environment (P). With *E. coli* inner membrane preparations, two discontinuities were observed at characteristic temperatures (t_l and t_h) irrespective of the spin label used. However, analysis of sealed animal cell membranes gave four characteristic temperatures when the hydrophobic label (5N10) was used, but only two with the amphiphilic spin label (12-NS-GA). The set of characteristic temperatures depends on the orientation of the membrane. When Newcastle disease virus propagated in embryonic chick eggs is used, the two characteristic temperatures are approx. 14 and 33°C, Newcastle disease virus budding from the surface of the host cell being considered as a source of right-side-out membranes. When latex phagosomes, which are inside-out membranes, are obtained from LM cells and assayed, the characteristic temperatures are 23 and 38°C. With unsealed or disrupted membrane preparations (sonicated phagosomes, LM membrane fragments attached to latex), both sets of characteristic temperatures are recorded. For example, with sonicated phagosomes, the temperatures are 12.7, 22.3, 29.2 and 38.3°C. It was concluded [354] that the inner and outer leaflets of animal cell membranes are physically distinct, each exhibiting a set of characteristic lower and higher limits of temperature for the lateral phase transition of lipids. It was envisaged that at all temperatures the outer membrane monolayer is probably less rigid than the inner monolayer and that this state could result from a lower protein : phospholipid ratio in the outer monolayer and/or the presence of phospholipids with lower melting points. The fact that the outer leaflet is richer in saturated phosphatidylethanolamines does not support this conclusion, but does not invalidate it.

Waggoner and Stryer [355] have synthesized different fluorescent probes showing affinity for different regions of a phospholipid molecule in liposomes as assayed with phosphatidylcholine dispersions. For example, 12-(9-anthroyloxy)stearic acid probes the hydrophobic region of the membrane. *N*-Dansylphosphatidylethanolamine probes the glycerol region of the phospholipid, while octadecylnaphthylaminesulfonate probes the water-exposed region of the phospholipid [355]. Different depths of bilayers have been examined using 8-anilinonaphthalene-1-sulfonic acid and 2-(9-anthroyloxy)palmitic acid, which bind close to the polar/apolar interface, and *N*-phenyl-1-naphthylamine and 12-(9-anthroyloxy)stearic acid [356,357]. Thus, the presence of a gradient of fluidity [346] towards the center of the bilayer has been confirmed. In other studies, a series of *n*-(9-anthroyloxy) fatty acids (n = 2, 6, 9 and 12) was synthesized as fluorescent probes which were found to be sensitive to the fluidity gradient of the lipid bilayer as assayed with dipalmitoyl phosphatidylcholine. Fluorescence quenching shows that these probes are located at progressive depths. Methyl-9-anthroate, which was also assayed, can be located

at the center of the bilayer. The largest blue shift observed in this experiment was that between 2-(9-anthroyloxy)palmitic acid and 6-(9-anthroyloxy)stearic acid, indicating that little water penetrates beyond the 6-(9-anthroyloxy)stearic acid position [358]. All the probes can detect the transition in the bilayer. Recently, fluorescent probes have been prepared containing the anthranoyl, dansyl or pyrene rings bearing acidic, basic or neutral functional groups and alkyl 'spacers' of various lengths [359]. Different anthracene or naphthalene derivatives can be used to investigate the phase transition temperature in phospholipid vesicles as has previously been observed by Overath and Träuble [360]. Values in agreement with those reported by other authors [361 -363] were obtained with phosphatidylcholine vesicles. Among these compounds (altogether 22 derivatives were synthesized and assayed), anthranoyl esters appear promising in the study of membrane asymmetry [359]. For example, the anionic derivative, 9-(2-sulfoethylanthroate), as well as a quaternary amine, triethylaminoethylanthroate, both of which are unable to flip from one bilayer of the membrane to another, the latter because of its fixed positive



9-(2-sulfoethylanthroate) triethylaminoethylanthroate dimethylaminoethylanthroate

charge, are crenators, whereas the tertiary amine, dimethylaminoethylanthroate, is a cup-former. When assayed with erythrocytes, these compounds act according to the bilayer couple hypothesis. However, when the number of carbon atoms of the spacer chain increased, the behavior of some molecules did not follow the predicted pattern. It was suggested that the most important determinant of the shape changes may be perturbations near the surface, the probes affecting the deeper regions not being expected to induce shape changes. 8-Anilino-1-naphthalenesulfonate, a widely used fluorescent probe, is a crenator [364] and in this respect follows the bilayer couple hypothesis. Studies on monolayers and liposomes using anthroyl derivatives of fatty acids also led to the conclusion that the perturbation caused when the bulky probe is located at the center of the bilayer is less than that when the probe is located near the surface [365].

The use of X-ray diffraction has led to controversy as to the distribution of cholesterol in myelin membrane leaflets. Some authors have presented electron density profiles which they interpret as indicating the asymmetric distribution of cholesterol [366]. However, using another choice of phase, a more symmetric profile has been suggested [367]. Wide-angle X-ray diffraction studies have led to the conclusion that in gram-negative bacteria outer membrane, part of the phospholipids from the fluid domains exists as a monolayer and/or as a bilayer [368]. The presence of the monolayer would be in keeping with the fact that in these bacteria, lipopolysaccharides are located only on the outer leaflet of the external membrane [25].

Recently, freeze-fracture techniques have found applications in the study of membrane asymmetry. Human red blood cells have been electrostatically linked in 'monolayers' to positively charged (polylysine-treated) glass and then fractured. The detached part, enriched in the external leaflet of erythrocyte membranes, and the bound part, enriched in the internal leaflet, were then chemically analyzed. It was found that the external leaflet is richer in cholesterol than the internal leaflet [369]. Freeze-etching tech-

niques have led to interesting conclusions as to the interpretation of particles observed in different membranes such as erythrocytes, bacteria, fiber cells of eye lens, gap junctions and luminal epithelial cells of the bladder, and particular significance was attributed to the presence or absence of complementary pits [370]. In general, the protoplasmic fracture face is richer in particles than the exoplasmic fracture face, although exceptions exist [371,372] (see also the companion paper [2]). Thus, with erythrocytes, it has been suggested that particles attributed to the transmembrane glycoproteins are mostly associated with the protoplasmic fracture face, without pits appearing on the exoplasmic face (for an interesting discussion, see Ref. 370), and that this is correlated with a stronger interaction of the protein with cytoplasmic elements and with proteins and/or phospholipids of the inner monolayer, these interactions pulling the integral proteins to the protoplasmic fracture face during fracturing. Upon etching, the external surface gives rise to holes and, as mentioned, the outer fracture face shows no depression. With gram-negative bacteria, the exoplasmic fracture face is very rich in particles (8000/μm^2) of diameter 40–80 Å and thus appears granular. The complementary inner fracture face of the outer membrane shows pits (6000/μm^2) of diameter 40–60 Å and a small number of particles of diameter 100 Å [373,374]. Some observations seem to indicate that in this case lipids rather than proteins may be responsible for the presence of particles [370]. This idea has been reinforced by studies with *E. coli* mutants lacking various proteins of the outer membrane [370,373–375]. In particular, although a mutant lacking the three proteins a, b and c showed only 25% of the granulated exoplasmic fracture face, incubation of these mutants with Ca^{2+} resulted in the appearance of particles densely covering this face and of corresponding pits on the inner fracture face [376]. On the other hand, lipopolysaccharide mutants [371–375] also show an important reduction in the granular surface. Furthermore, when half of the lipopolysaccharides of *E. coli* was eliminated by treatment with EDTA, 50% of the outer membrane exoplasmic fracture face particles and 50% of the pits of the outer membrane 'protoplasmic' fracture face disappeared [374]. Similar results have been reported for *Pseudomonas aeroginosa*, although in this case, part of the cell envelope proteins is also extracted [377,378]. However, in different lipopolysaccharide mutants of *S. typhimurium*, the particle density correlated well with the amount of outer membrane proteins [379]. It has been postulated that the particles may be constituted of lipopolysaccharide aggregates stabilized by divalent cations and proteins [370,376] and may represent transmembrane pores, the specificity of which is dependent on proteins [373,376]. That the outer membrane exoplasmic fracture face is particle-rich may correlate [370] with the participation of lipopolysaccharides which are known to be confined to the outer leaflet [25]. However, the outer leaflet membrane in gram-negative bacteria is also protein-rich [380–382]. Nonetheless, other observations have been interpreted as supporting the hypothesis of the lipid origin of particles when complementary pits are observed [370]. For example, in the luminal membrane of bladder epithelial cells, as for *E. coli* outer membrane, most of the particles are found on the exoplasmic fracture face, complementary pits appear on the protoplasmic fracture face [383] and these particles together with their corresponding pits disappear upon treatment with either phospholipase or lipid solvents [384]. Gap junctions [385–398] generally show particles on the protoplasmic fracture face and pits on the exoplasmic fracture face. In Arthropoda, the localization of particles and pits is reversed [389]. In this case, also lipids rather than proteins have been suggested to give rise to the particles [370]. Complementarity is also observed in fiber cells of eye lens [391] and this, in contrast to other studies [392], has been explained on the basis of the changes of the lipid environment on aging leading to

aggregation of intramembraneous particles rather than formation of gap junctions. Verkleij and Ververgaert [370] have found similarities between the gap junctions and the outer membrane of gram-negative bacteria, on the basis that, in both [399,400] cases, membranes are permeable to ions and small molecules. On the other hand, subunit patterns are observed using negative staining of both the outer membrane of *E. coli* [401] and gap junctions [385]. The authors [370] question whether significance should be attributed to the nature of lipids involved in the composition of junctions and in particular to the globosides which were suggested to be involved in the formation of neuromuscular junctions [402].

IX. Conclusions and discussion

I have briefly reviewed the techniques used for assessing the distribution of membrane lipids. It is clear that, in view of the many contradictory and often poorly explained divergent data, one must be cautious in interpreting the results. Experimental conditions play a crucial role and hence, rearrangements can take place. Thus, lipopolysaccharides of gram-negative bacteria have been reported to be symmetrically distributed [26,27]. Under milder conditions, they were found to be asymmetrically located [25] with the sugar moiety oriented towards the medium. 'Annealing' (for example, working at 45°C) increased the amount of phosphatidylinositols detectable by specific antibodies in myelin and microsomal membranes from 15 to 50% of the membrane content [49]. In many circumstances, proteolytic digestion may increase the reactivity of lipids. This is observed when antibodies, lectins, modifying enzymes or chemical reagents are used. For example, proteolytic treatment of mitochondria and microsomes increases the adsorption of anti-phosphatidylinositol antiserum [50]. A significant observation is that anti-phosphatidylglycerol antibodies react with *Mycoplasma hominis*; however, cells are not agglutinated unless partial hydrolysis of membrane proteins is carried out [52]. This situation is reminiscent of the case I reported in the companion paper [2], that of the ferredoxin-NADP oxidoreductase in chloroplast thylakoid membranes [403,404] in which crypticity is observed. Antibodies raised against this enzyme can inhibit it, indicating that its active site is exposed to the chloroplast stroma. However, the precipitation of membranes does not take place unless the chloroplast coupling factor (CF_1) is eliminated by treatment with EDTA. In *M. hominis*, phospholipase C from *B. cereus* fails to attack the phospholipids of intact cells or isolated membranes, although it readily hydrolyzes extracted lipids. After partial proteolysis, phospholipase C becomes active. The action of *B. cereus* phospholipase C on lipopolysaccharide-producing strains (S or R_c type) of *S. typhimurium* has shown the inaccessibility of the enzyme to phospholipids [206] with strains producing defective lipopolysaccharides (R_d or R_e type) and only a reduced amount of proteins, phosphatidylethanolamines could be hydrolyzed. These results have been confirmed by the action of CNBr-activated dextran, used as a non-penetrating reagent, on aminophospholipids. It was concluded that the outer membrane of S and R_c strains either contains all the phospholipid molecules in its inner leaflet or that heads groups are completely covered.

Fetal erythrocytes as well as trypsinized erythrocytes react with antibodies to tetrahexosylceramides (globosides). In normal erythrocytes, even Forssman antigen (with five sugar residues) does not react with the corresponding antibodies, indicating that at least six residues are necessary in the sphingolipid for the accessibility of antibodies [112,114, 116,117]. This case is of particular interest, since reaction with galactose oxidase-

$NaB[^3H]H_4$ labeled not only tetrahexosylceramides but also trihexosylceramides [115], demonstrating the differential accessibility of antibodies and of the enzyme, galactose oxidase. Some species of Mycoplasma such as *M. laidlawii* and *M. fermentans* were found to be devoid of lectin binding sites at the cell surface. Pronase digestion uncovered hidden glycolipids able to react with lectins [58,60]. A note of caution seems appropriate at this point; proteolytic treatment, as discussed in the companion paper [2], may have effects other than the simple unmasking of molecules and complex interplay with the process of lipid metabolism has been envisaged in some cases [405,406].

The reactivity of a chemical reagent is influenced by surface molecules. In En(a$^-$) erythrocytes, which lack glycophorin, and in heterozygous En(a) erythrocytes, which are deficient in glycophorin, the labeling of aminophospholipids with trinitrobenzenesulfonate was found to be enhanced [93]. According to the metabolic state, the accessibility of phospholipids to reagents may vary. In ATP-depleted erythrocytes, 45% rather than 20% of the phosphatidylethanolamines are labeled by trinitrobenzenesulfonate [177]. Other problems with reagents are encountered. A reagent known to be nonpermeant may be permeant according to the circumstances. Thus, many anionic compounds have been reported to penetrate the erythrocyte membrane [90,91]. Trinitrobenzenesulfonate penetrates the membrane of *B. megaterium* at 15°C but not at 0°C [10]. The reaction with this reagent was reported not to reach completion; the bulkiness and the charge of the reagent have been suggested as the cause of incomplete reactions [23,75,95]. However, the completion of labeling of phosphatidylethanolamines has been observed under certain conditions [66,67] and it has been reported that for vesicular stomatitis virus, the disruption of the membrane renders all the phosphatidylethanolamines reactive to trinitrobenzenesulfonate [78]. In some cases, the incomplete reaction of phosphatidylethanolamines was also explained on the basis of the strong interaction of the lipid with proteins [80]. An observation correlated with the sidedness of aminophospholipids in erythrocytes is that the oxidation of spectrin, an internal cytoskeletal element forming a peripheral membrane protein, leads to appreciable accessibility of these lipids to interaction with trinitrobenzenesulfonate and phospholipase A_2 [148,149].

Studies with mitoplasts of rat liver which are right-side-out particles, and ETP_H particles from bovine heart mitochondria which are inside-out particles, using trinitrobenzenesulfonate and fluorodinitrobenzene have been conducted at two different pH values, 8.2 and 8.5 [67]. Both particles appear more stable in pH 8.2 buffer and very little hydrolysis of phospholipids occurs. In pH 8.5 buffer, extensive degradation of dinitrophenylated and trinitrophenylated phosphatidylethanolamines and lysophosphatidylethanolamines occurs and dinitrophenyl glycerophosphorylethanolamines and trinitrophenyl glycerophosphorylethanolamines are formed. Dinitrophenyl glycerophosphorylethanolamines and trinitrophenyl glycerophosphorylethanolamines are also degraded by a phosphodiesterase to dinitrophenyl ethanolamine and trinitrophenyl ethanolamine. When ETP_H particles are labeled with trinitrobenzenesulfonate and fluorodinitrobenzene, washed and incubated in pH 8.2 and 8.5 buffers, the resynthesis of trinitrophenyl phosphatidylethanolamines and dinitrophenyl phosphatidylethanolamines is observed in the first buffer by means of acylation of trinitrophenyl lysophosphatidylethanolamines and trinitrophenyl glycerophosphorylethanolamine or dinitrophenyl lysophosphatidylethanolamines and dinitrophenyl glycerophosphorylethanolamine. In pH 8.5 buffer, trinitrophenyl phosphatidylethanolamines are degraded to trinitrophenyl lysophosphatidylethanolamines whereas dinitrophenyl phosphatidylethanolamines continue to be synthesized from dinitrophenyl glycerophosphorylethanolamine. These observations were taken

to indicate that phospholipases, phosphodiesterases and acyl transferases are at work and can modify the physionomy of immediate labeling products [67].

The use of chemical reagents has the inherent drawback that specific lipid classes are recognized. Careful use of the degradation enzymes allows the attack of most lipid classes. However, caution should be exercized in using this technique. The reported specificity of phospholipases [133–144,162,209] should be borne in mind. Therefore, if a particular enzyme does not hydrolyze a given phospholipid in right-side-out biomembranes, one cannot automatically deduce its location on the inner leaflet. Moreover, even a phospholipase C specific for phosphatidylinositols, which has proved to be active either on extracted lipids or detergent dispersions of microsomes, does not hydrolyse phosphatidylinositols in either intact or leaky microsomes [162]. Similarly, *C. perfringens* sialidase attacks partially and the enzyme from *V. cholerae* does not attack sialic acid residues linked to sialoglycolipids in murine melanoma B16 tumorigenic cells [129]. The action of phospholipase A_2 on erythrocytes has been reported to depend on Ca^{2+} and pH [23,147] and that on erythrocytes [147,175,177] and *A. laidlawii* [178] to depend on the metabolic state. Enzyme degradation, as observed with the action of phospholipase A_2 on either lecithin vesicles [179] or *M. laidlawii* [180], also distinguishes between molecules in the fluid or the gel state. The temperature dependence of hydrolysis of the phospholipids of *B. subtilis* by *B. cereus* phospholipase C was similarly attributed to the phase separation at low temperature [75].

An important point with regard to the action of enzymes on lipids is that enzymes change the nature of their substrates and, particularly, the favored arrangement of reaction products may be different from that of the substrates. For example, as mentioned above, the action of sphingomyelinase on erythrocyte membranes promotes the formation of 60-Å particles and pits on freeze-fracture surfaces, this appearance revealing the formation of inverted micelles of ceramides [186]. Important changes induced in membranes as a result of enzyme action led different authors to ensure that lysis did not occur, since the enzyme responsible would then have had access to both sides. I have mentioned different methods used to test whether lysis occurs during enzyme action [17, 76,155,162,248,267]. On the other hand, even without lysis, flip-flop of molecules may be promoted. The possibility of the transbilayer movement of lipids as a result of enzyme action has been envisaged in different cases such as with *B. subtilis* [207], *B. megaterium* [208] and *M. laidlawii* [168].

The removal of products of the reaction with phospholipase A_2 has been reported to protect intracellular organelles from lysis [155] and to facilitate hemolysis of erythrocytes [176]. The source of the enzyme is important; although phospholipases A_2 of different origin have been reported not to cause lysis of erythrocytes, a basic phospholipase A_2 elicited hemolysis under certain conditions [147].

I have discussed the striking discrepancies in the results on the sidedness of lipid distribution in endoplasmic reticulum. Sundler et al. [157], who used phospholipase A_2 from *C. atrox* and phospholipase C from *B. cereus*, suggested that the results of Nilsson and Dallner [155,156], obtained using phospholipase A_2 from *N. naja*, may be erroneous and might reflect the specificity of the enzyme which the latter authors used. Higgins and Dawson [162] have found that both phospholipase A_2 from *N. naja* and phospholipase C from *B. cereus* are not suitable, since both damage the vesicles. Different cases of lysis have been reported by other authors when using phospholipase A_2 [164–167] and the influence of temperature on the amount of lipids hydrolyzed has been noted [18]. However, an agreement as to the causes of the divergent results on microsomes does not exist.

The action of phospholipase C also has been found to be dependent on the metabolic state of erythrocytes [175,177,188]. On the other hand, in different experiments, the influence of temperature was interpreted in terms of phase separation [75,208] and the transbilayer movement of lipids was envisaged as a consequence of perturbation of the lipid composition of the exposed leaflet of the membrane [75,168,207,208]. The action of sphingomyelinase on erythrocyte membranes has been reported to promote the formation of particles and pits which appear on the fracture faces of erythrocyte membranes [186] due to the arrangement of ceramide molecules in an inverted micellar form [370]. Another problem is the failure of completion of the enzyme action to occur, even if both leaflets of a leaky membrane are exposed. In such cases, the strong interaction of lipid with proteins is suggested to be the cause, as with chromaffin granules, in which 20% of the lysophospholipids were found not to be attacked by a lysophospholipase [216].

It has been assumed, as in the assignment of lipids to both leaflets of the erythrocyte membrane, that lipids are in an approximately equal distribution between the cytoplasmic and the exoplasmic leaflets. In contrast, it may be that the opposite holds true [76,211]. For example, in *B. amyloliquefaciens*, the distribution of lipids was reported to be very unequal; 70% of the total lipids being localized on the outer leaflet. Conversely, 75% of the membrane proteins were estimated to be associated with the inner leaflet [76].

Concerning the use of exchange proteins in investigations of the distribution of membrane lipids, the basic assumption is that only the outer leaflet lipids are exchanged [229, 243–248]. Consequently, lipid exchange has been used for the study of the distribution of lipids in membranes [17,18,69,210,211,243,248,250,255–257,265] and the technique has been employed for the in situ manipulation of the membrane protein environment [169,266,270–272]. However, using exchange proteins, rapid transbilayer movement has been observed with microsomal and mitochondrial [17,18] preparations. It was ascertained that the permeability of microsomes is not affected and that the vesicles formed by extracted lipids consist of two distinct pools of lipids [17,18]. Even in vesicles, unilateral modification of the nature of the lipids either by the use of exchange proteins [273] or by enzyme modification [22] led to rapid transbilayer movement. Furthermore, we have seen that the results of enzyme degradation of some bacterial membranes are explained on the basis of flip-flop of phospholipids induced as a result of unilateral modification [75,168,208]. For cholesterol, rapid flip-flop has been reported for erythrocytes [276–278] and vesicular stomatitis virus [279], whereas a slow rate of transmembrane movement has been observed for influenza virus [210] and *M. galliseptum* [280]. Even with lipid vesicles, contradictory results on the rate of cholesterol flip-flop have been reported [252,281–283].

The presence of the erythrocyte membrane integral protein, glycophorin, has been observed to enhance the lipid flip-flop rate in simple vesicles [284,285]. However, when cytochrome oxidase, a membrane-spaning complex from the inner mitochondrial membrane, was incorporated into vesicles, the rate of transmembrane movement of lipids did not change [286], so that there is no general rule implying the participation of integral proteins in the flip-flop movement of lipids.

Apart from results on the unequal distribution of lipid molecules in the two leaflets of artificial vesicles [288–294], NMR techniques provide a tool for the estimation of the nature of lipid arrangement, whether bilayer, hexagonal type II or intermediary phases [20,21,299,319]. ^{31}P-NMR spectra of bovine and rat [15] and rabbit [16] microsomal

fractions have led to the suggestion that a part of the lipids may experience isotropic motion probably due to the presence of inverted micellar or short cylindrical hexagonal type II arrangements. In sarcoplasmic reticulum, both ^{1}H-NMR [19] and ^{31}P-NMR [299], and in inner mitochondrial membrane ^{2}H-NMR [331] and ^{31}P-NMR [299], indicated the presence of non-bilayer arrangements.

Freeze-fracture studies carried out on extracted lipids of mitochondrial membrane [327], rod outer segment [332] and *E. coli* [333] have demonstrated the presence of lipid particles and pits, indicating the presence of the inverted micellar arrangement 'sandwiched' between bilayer phases. However, the presence of particles on the 'exoplasmic' fracture face and pits on the protoplasmic fracture face of the outer membrane of gram-negative bacteria has been attributed to lipopolysaccharides present in the outer leaflet of this membrane, or to aggregates of lipopolysaccharides stabilized by divalent cations and proteins [370,376]. Different cases, such as the luminal membrane of bladder epithelial cells, some gap junctions and fiber cells of eye lens have been reported in which particles are present on the exoplasmic fracture face and pits on the protoplasmic face. It has been suggested that the particles may represent lipids with an asymmetric distribution rather than proteins [370]. Reports on the particles and pits observed during freeze-fracture studies of lipid extracts of biological membranes [327,332,333] confirm the existence of lipid particles attributed to the presence of a non-bilayer arrangement of lipids. It remains to be elucidated how the asymmetric distribution of lipids can give the same freeze-fracture appearance.

I have previously discussed the question as to the origin of unequal distribution of lipids in the membrane [1] and envisaged as an important contributing factor the asymmetry of the environment of both sides of biological membranes. The inequality of the distribution of lipids was considered to be the outcome of the rates of processes which tend to increase this distribution and of those which tend to dissipate it. These factors should be sought objectively, since they depend on the nature of the membrane examined and, probably, for a given membrane also on the developmental step and physiological state. As suggested [1], the role of the environment is many-fold and embraces all the causes of asymmetric lipid perturbations. The distribution of ions [13,14,407,408], proteins [13,14], enzymes of both synthesis and degradation, exchange proteins and induction of a non-bilayer arrangement, even if local and transient, are possibilities which should be considered. Of course, the role of membrane proteins, both peripheral (as exemplified with spectrin [148,149]) and integral, and that of selective flow should also be envisaged [1]. Depending on the particular case, each factor may be of greater or lesser importance. One should consider constantly not only what constitutes a membrane but also its environment which, being asymmetric, influences membranes asymmetrically: the concept of the membrane environment was emphasized [1]. Some recent developments are worthy of note. It has been reported that in rat erythrocytes, exchange of phosphatidylcholines [409] is responsible for the renewal of the more unsaturated species of the outer leaflet and that acylation activity is directed towards the formation of diunsaturated phosphatidylcholines at the inner face [410]. On the other hand, two methyltransferases have been reported to be involved in the genesis of phosphatidylcholines in erythrocyte membrane. The first enzyme, transferring a methyl group to phosphatidylethanolamines, is located in the inner surface, the second, which transfers methyl groups to phosphatidyl-*N*-monomethylethanolamines, facing the exterior. Substrates of the first enzyme (phosphatidylethanolamines) are localized on the cytoplasmic face and the products of the second enzyme are mostly found on the outer surface [411]. The intermediates, phosphatidyl-*N*-

monomethylethanolamines, are purported to be embedded in the membrane. Similarly, microsomes and synaptosomes of rat brain were reported to form phosphatidylcholines by methylation of phospholipids [412]. In synaptosomes, a methyltransferase (methyltransferase I) catalyzes the methylation of phosphatidylethanolamines to phosphatidyl-*N*-monomethylethanolamines. This enzyme was found to be tightly bound to the membrane. The second methyltransferase (methyltransferase II) catalyzes the two successive methylations to yield phosphatidylcholines. This enzyme was found to be easily solubilized by sonication [412]. In another study on the genesis of phosphatidylcholines in microsomal membranes using either in vivo incorporation of [^{14}C]choline or CDP-[^{14}C]-choline as a substrate for in vivo assays, it was concluded from degradation studies with *C. perfringens* phospholipase C that phosphatidylcholines from the outer leaflet are preferentially labeled by the choline-phosphotransferase pathway and that this pool does not equilibrate with that of the inner leaflet. The inner leaflet phosphatidylcholines have been suggested to be synthesized by methylation of phosphatidylethanolamines [413]. Furthermore, one of the various distributions suggested for lipids in microsomal membranes [162] has been confirmed and, in particular, phospholipases from bee venom and *N. naja* have been confirmed to cause leakage of the contents of microsomes [413].

In closing this section, I wish to mention that one should be aware that techniques used for the study of the unequal distribution of membrane lipids have been most often aimed at obtaining information as to the ratio of the molecules on the leaflets. The nature and distribution of these molecules, compatible with the maintenance of life, may be more or less modifiable and change according to the experimental conditions as exemplified throughout this article and, probably, according to physiological and physiopathological conditions or developmental steps for a cell [1]. A question may now be raised: does the inequality of lipid distribution, as revealed, always reflect the best fit for the function of individual proteins? In other words, do cases exist in which the function of a protein in a given leaflet is best modulated by a lipid which forms only a minor fraction in that leaflet?

Acknowledgements

I wish to thank my wife Raymonde for her indispensable assistance during the preparation of this and the companion manuscript. Financial support during this work was given by Centre National de la Recherche Scientifique and Délégation Générale à la Recherche Scientifique et Technique (Grant No. 77.7.1287).

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