Chem. Phys. Lipids 8 (1972) 366–373 © North-Holland Publishing Company

PERMEABILITY AND TOPOGRAPHY OF MEMBRANES

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In this brief review, we will report that the permeability of both artificial and natural membranes depends at least on the following lipid parameters:

(I) The nature of the hydrocarbon chains of phospholipids. Because of a less compact packing of unsaturated phospholipids when compared with saturated ones, the former barrier will be more permeable.

(II) Interaction of phospholipids with sterols. The so-termed condensing effect of a sterol like cholesterol limits the mobility of the fatty acid chains. The effect depends not only on the chemical nature of the phospholipid, but also on structural details of the sterol partner.

(III) The chemical nature of the polar headgroup of the phospholipid. Wide variations in structure occur and the net charge of phospholipid can range from extremely negative to positive, thereby influencing ion permeation.

In addition some recent results obtained with action of pure phospholipases A and C on erythrocytes will be mentioned.

I. The fatty acid constituents of phospholipids

Conspicuous differences in physical properties have been detected between various phospholipid species. In our laboratory research was concentrated on the behavior of the synthetic phospholipids in monomolecular layers and in bilayers with the hope of obtaining information about the contribution of phospholipids to the barrier properties of biological membranes.

In a monomolecular layer at the air-water interface the area occupied by a lecithin molecule appears to depend very much on the nature of the hydrocarbon chains. When the saturated hydrocarbon chains become shorter, there is a decrease in London-Van der Waals forces allowing a greater mobility of the chains. The same is true for the introduction of unsaturated fatty acid constituents demonstrating that the space occupied by a phospholipid molecule increases with increasing unsaturation[1–3]).

Extrapolating from the monolayer results one can expect that the permea-

tion through a lipid bilayer will very much depend on the species of lecithin present. To verify this assumption, use was made of liposomes. Both the penetration rate across these bilayers and the leakage from liposomes were measured [3-5]). Using liposomes made of various synthetic lecithin species it could be demonstrated that a decrease in chain length caused a higher permeability of the lipid bilayer for compounds such as glycerol and glucose. The permeation of these non-electrolytes through the lipid-barrier becomes easier when the degree of unsaturation of the lecithin species increases. There is an obvious correlation between the packing of molecules in the monolayer and artificial bilayer. While the phospholipid species in the bilayer dictate the barrier properties, the crucial question is, if we may extrapolate to biological interfaces. In other words, is the permeability behavior of natural membranes dependent on the nature of the fatty acid constituents of membrane lipids?

For several membranes investigated to date the answer is yes. A good example is provided by *Mycoplasma laidlawii*. In this micro-organism the fatty acid composition can be varied within rather wide limits by supplementing the different fatty acids to the growth medium. On intact cells and liposomes prepared of lipids extracted from these cells measurements of the permeability were carried out [6, 7]). Both the natural membranes and the artificial bilayers showed an increase in permeability with increasing unsaturation. However, with increasing number of double bonds of the unsaturatd fatty acid, the rate of incorporation decreases. The order is monoenoic > dienoic > trienoic > tetraenoic, while a hexaenoic acid is hardly incorporated at all [7]). We can conclude that the membrane properties are controlled here at the level of phospholipid biosynthesis so as to prevent the formation of leaky membranes.

The preceding experiments on passive diffusion raise the question whether the lipid composition in a membrane may also regulate facilitated diffusion. This problem was studied using valinomycin as a model for carrier transport. The action of valinomycin was studied on liposomes made-up of different lecithins. The spontaneous leak of radioactive Rb^+ is very low. The addition of a very small amount of valinomycin gives a significant stimulation of transport and the effect turned out to be highly dependent on the nature of the fatty acid chains of the phospholipids. The more unsaturated lipids allow for a higher mobility and efficiency of the carrier [8]). Very recently it could be demonstrated that this phenomonon occurs in a natural membrane as well. Mycoplasma cells containing more unsaturated fatty acid constituents appear to allow for a more efficient exchange of K^+ catalyzed by valinomycin [7]). These results suggest that the nature of the lipid is not only important for simple diffusion but may also control the rate of carrier mediated trans-

port. In a complex biological membrane, two carriers with a high and a low working-gear should be located in an unsaturated and a saturated lipid locus, respectively.

II. Phospholipid-sterol interaction

Another factor which appears to be important for the stability and permeability of membranes is the sterol content. This lipid parameter varies widely. The role of sterols has been studied extensively in mono and bilayers. It is well established that a sterol like cholesterol, when spread together with a natural lecithin, at the air-water interface, gives, as an overall effect, a reduction of the mean molecular area of the lecithin molecules. This observation raises several questions: (a) Is the condensing effect dependent on the nature of the phospholipid species?, (b) Which structural features of the sterol are important for this action?, (c) Is this phenomenon relevant to biological membranes? Using synthetic lecithin species, it was found that indeed the so-termed condensing effect or ordering effect and its magnitude depends on the fatty acid constituents and their pairing within the lecithin molecule [1-3]. Without going into all details, it can be concluded that at least all natural phospholipid species having one unsaturated fatty acid constituent reveal reduction in their molecular size in a monolayer in the presence of cholesterol. One would expect that those lecithin species which are at the air-water interface would display similar behavior in a lipid bilayer. This is indeed the case as demonstrated by the decrease in permeability of liposomes in which cholesterol was inserted [3, 9]. Also in the natural barriers, cholesterol reduced the permeability as was proven in a different manner. Cells of *Mycoplasma laidlawii* grown in the presence of cholesterol appeared to have a lower permeability than those not containing sterol [6, 7, 11].

A second approach is quite different. Erythrocytes can be forced to loose part of their membrane cholesterol just by simple incubation with liposomes. It was found that these cells which are depleted by some 30–40% of their membrane cholesterol become permeable and also less stable [10].

Not only the structural details of the phospholipids, but also those of the sterol are important. This can be demonstrated by replacing the cholesterol of the erythrocyte by analogs [10] or by incorporation of different sterols into Mycoplasma [11]. At the same time, one can study the behavior of the particular sterol in monolayers and bilayers with an appropriate phospholipid partner [12]. At this stage of the investigations it is concluded that the reduction of membrane permeability caused by cholesterol is dependent on a planar sterol nucleus, an intact side chain and the presence of a 3-β-hydroxy group in the sterol.

III. The polar headgroup of phospholipids

That the nature of the polar headgroups can affect the permeability properties of a natural membrane could be demonstrated in bacteria [13]). Two important phospholipids of gram-positive bacteria are phosphatidylglycerol and a derivative denoted lysylphosphatidylglycerol. The first has a net negative charge and the second is positively charged. The ratios between both phospholipids in the membrane can vary significantly dependent on the environmental conditions. In agreement with previous studies of lipid bilayers showing that the net charge of the interface has a significant effect, it was found that artificial barriers of phosphatidylglycerol were cation and proton selective whereas those of lysylphosphatidylglycerol were anion selective. For non-electrolytes, the permeation through bilayers of lysylphosphatidylglycerol is easier than through phosphatidylglycerol. The former has a more bulky polar headgroup as shown also on monolayers and prevents close packing of the chains which are similar in both phospholipids. Thus the effect on ion permeability is dependent on the charge of the phospholipid. It was found that valinomycin is highly active as a carrier throughlipid bilayers negatively charged of phosphatidylglycerol. But, its action appears to be immobilized when the bilayer exists of lysylphosphatidyl glycerol [13]).

These observations on liposomes could be extended to membranes of *Staphylococcus aureus*. The ratio of phosphatidylglycerol to lysylphosphatidylglycerol was varied, this depending on the time of exposure to glucose medium of pH 5. It was found that cells with dominant lysylphosphatidylglycerol content were, just as liposomes, more permeable to non-electrolytes. Measurements of the action of valinomycin showed that there is a striking reduction in the ability of this carrier with increasing ratio of the positively charged lysylphosphatidylglycerol to the negatively charged phosphatidylglycerol [13]). Again, a magnitude of carrier-facilitated transport appears to reflect the significance of the lipid composition just as in artificial membranes.

At several instances we have seen not only that the permeability of a natural membrane depends on details of the lipid constituents but also that there is a similarity between the natural membrane and artificial bilayers. This certainly is not meant to be translated as meaning that membranes are built up of bilayers of lipids exclusively. The approaches made in our laboratory had the purpose of illustrating only that the chemical structures of phospholipids and sterols have consequences for membrane properties. These and many still unknown details are needed to understand the interaction of lipids and protein so as to come in the future to better molecular descriptions of the biological membrane.

In order to obtain some information about the topographical make-up of the lipid region of membranes, studies are carried out on the action of various completely pure phospholipases on defined ordered lipid systems and a variety of biological membranes. Confining the present discussion to recent results on erythrocytes [14]), it can be stated that phospholipase A_2 (from porcine pancreas) and phospholipase C (from Bacillus cereus) do not lyse intact human erythrocytes and produce no breakdown of the phosphoglycerides in the native membrane. By contrast, phosphoglycerides in erythrocyte ghosts (but not sphingomyelin) are degraded. This indicates that more caution, than is usually exercised in the literature, is necessary when results obtained on isolated membranes are used to explain the detailed molecular structure of the native membrane. Perturbations of the erythrocyte membrane which do not themselves cause lysis of cells, appear to render the phospholipids susceptible to attack by these phospholipases.

Acknowledgments

The author expresses gratitude to all colleagues who contributed to this research program.

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Discussion

WALLACH commented on the susceptibility of everythrocyte membranes to the attack by pure enzymes. Whilst the membrane of ghosts is very sensitive to proteases and also to phospholipase C, this was not so with inverted ghosts which are very resistant to protease action, but also quite susceptible to purified phospholipase C.

VAN DEENEN said this was an interesting comment and they had not so far studied such inverted membranes.

BANGHAM mentioned some experiments where the carrier property of valincomycin was reduced by the presence of saturated fatty acids in the membrane. He wondered whether this was due to a reduction of the mobility of the valinomycin, or to a reduction of its partition coefficient.

VAN DEENEN said that as yet they did not know how to determine the partition coefficient for this system.

DEUTICKE commented that they had evidence that the phospholipids have something to do with the membrane permeability in higher animals. From their results, they concluded that there was a positive correlations between the unsaturation of the fatty acids and the permeability of anions On the other hand, a depletion of 40% of the cholesterol in pig red cells did not influence the permeability to phosphate, lactate and other organic anions. This was in agreement with Dr. Seeman's results about water permeability and cholesterol content, which influenced, in many cases, the permeability of non-electrolytes.

VAN DEENEN thought that the anions are probably going through special locations and cholesterol is located in different positions.

PELZER pointed out that according to Rodbell, incubation of isolated fat cells with phospholipase C had an insulin-like effect, obviously as a result of depletion of phosphorylcholine residues in the phospholipids. He asked whether the mechanism of action of insulin had anything to do with the conformation of the phospholipids.

VAN DEENEN replied that Dr. Rodbell had told him that this insulin-like action which was first observed was later considered to be not very relevant. The presumption of Dr. Rodbell originated from the membrane model of Dr. Lucy, according to whom a limited degradation of lecithins to diglycerides should transfer the bilayer structure into a micellar structure, resulting in holes for glucose to penetrate. Insulin did not induce glucose diffusion through the membrane of phospholipid liposomes. He thought that the action of insulin was surely more complicated.

LUZZATI pointed out that in recent papers by Bretscher it was implied that the structure of the membrane of ghosts and erythrocytes was identical, especially in respect to the glycoproteins. With regard to the results described by Van Deenen on ghosts and cells, surely this picture was now untenable.

WALLACH replied to this and pointed out that the glycoproteins had been studied very thoroughly recently. There were several non-permeant reagents, which in intact erythrocytes reacted not only with the major glycoproteins but reacted with all proteins in isolated ghosts. Similarly, proteases rapidly cleaved the proteins of isolated ghost membranes, but not those of intact erythrocytes.

VAN DEENEN said that the composition of ghosts could be very different according to the preparation method. He had prepared ghosts with the procedure of Dodge and Hanahan, and obtained a lipid:protein ratio of 1:1, whereas with the Parpart method it was 3:7. From this, it could be concluded that during each of the above preparations something was washed away and, therefore, a ghost membrane could not be compared with the membrane of the intact cell.

METCALFE wondered whether phospholipases were acting only from the outside, or whether they could penetrate to attack the ghost membrane also from the inside.

VAN DEENEN replied that with phospholipase C from *Bacillus cereus*, which is highly specific for phosphoglycerides, he had obtained the same hydrolysis kinetic with ghosts and with phosphoglycerides liposomes. Phospholipase C from *Clostridium welchii* also split sphingomyelins and, therefore, showed different kinetics. As only a few phospholipid molecules had to be hydrolyzed by the phospholipases to distintegrate the membrane structure, it was hard to decide whether or not the enzyme attacked the lipids from the inside of the ghost as well.

WALLACH said that he had produced normal oriented vesicles by exocytosis, as well as reversed vesicles by endocytosis of ghosts. He had found that the proteins of the former were susceptible to proteases, particle bound or not, whereas those of the reversed bags were not. At least these protease should, therefore, not penetrate the membrane. Similar experiments with phospholipases did not show any difference.

HASSELBACH asked Van Deenen if he had any idea how cholesterol esters were arranged in bilayers, as they were very important for the permeability of biological membranes.

VAN DEENEN replied that in most membranes he had worked with, he had always found only unesterified cholesterol. For instance, the erythrocyte membranes did not contain cholesterol esters, but these were very enriched in the serum as a part of the lipoproteins. The cholesterol of the erythrocyte

membrane was in equilibrium with the cholesterol of the serum lipoproteins, but apparently the cholesterol esters of the serum lipoproteins could not enter into the membrane. It was known that certain membranes contained cholesterol esters, for example, myelin membranes from patients with multiple sclerosis.

HASSELBACH commented that there were also small amounts of cholesterol esters in cytoplasmic reticulum and he proposed that they had something to do with permeability.

DA SILVA asked if traces of haemoglobin, which had not been extracted by preparing the erythrocyte membrane, imitated a resistance to phospholipases?

VAN DEENEN replied that haemoglobin traces should not have an influence as he always started the enzymic degradation from the outside.

KAMAT asked if it was possible to get ghosts with different contents of haemoglobin, and commented that maybe a critical haemoglobin content did mimic a certain degree of resistance of the membrane to degradating enzymes.

VAN DEENEN said he thought it was possible, but he had never done such experiments.