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INTERACTIONS IN LIPID-WATER INTERFACE ASSESSED BY FLUORESCENCE SPECTROSCOPY

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Abbreviations

A_m	=	molecular area
CF	=	carboxyfluorescein
CHOL	=	cholesterol, cholest-5-en-3β-ol
di-8-ANEPPS	=	4-[2-[6-(dioctylamino)-2-naphthalenyl]ethenyl]-1-(3-sulfopropyl)-pyridinium
DPH	=	diphenylhexatriene
DPPC	=	1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine
DPPN	=	1,2-dipalmitoyl-*sn*-glycero-3-phospho-[N-(4-nitrobenz-2-oxa-1,3-diazole)-ethanolamine]
GABA	=	γ-amino butyric acid
$GABA_AR$	=	type A GABA receptor
$GABA_C\rho_1R$	=	type C ρ_1 GABA receptor
GP	=	generalized polarization
IC	=	internal conversion
ICT	=	internal/intramolecular charge transfer
ISC	=	intersystem crossing
Laurdan	=	6-lauryl-2-dimethylaminonaphthalene
LGIC	=	ligand-gated ion channel
LUV	=	large unilamellar vesicle
MD	=	molecular dynamics
MLV	=	multilamellar vesicle
NBD	=	4-nitrobenz-2-oxa-1,3-diazole
NBD-PC	=	1-acyl-2-[12-[(7-nitro-2,1,3-benzoxadiazol-4-yl)amino]dodecanoyl]-*sn*-glycero-3-phosphocholine
OP	=	original publication
PC	=	phosphatidylcholine a.k.a. lecithin a.k.a. glycerophosphocholine
POPC	=	1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine
PREG	=	pregnanolone, 5β-pregnan-3α-ol-20-one
Prodan	=	6-propionyl-2-dimethylaminonaphthalene
S_0	=	electronic ground state
S_1	=	first excited electronic state
T_m	=	main phase transition temperature
VDAC	=	voltage-dependent anion channel
VR	=	vibrational relaxation
ΔX	=	change in property X
γ	=	surface tension
ν	=	vibrational energy level
π	=	surface pressure
Ψ	=	dipole potential
Ψ_0	=	monolayer dipole potential extrapolated to zero lipid surface density

List of original publications

I Alakoskela, Juha-Matti I. and Paavo K. J. Kinnunen. 2001. Control of a redox reaction on lipid bilayer surfaces by membrane dipole potential. *Biophys. J.* 80: 294–304.

II Alakoskela, Juha-Matti I. and Paavo K. J. Kinnunen. 2001. Probing phospholipid main phase transition by fluorescence spectroscopy and a surface redox reaction. *J. Phys. Chem. B* 105: 11294–11301.

III Söderlund*, T., J.-M. I. Alakoskela*, A. Pakkanen and P. K. J. Kinnunen, 2003. Comparison of the effects of surface tension and osmotic pressure on the interfacial hydration of a fluid phospholipid bilayer. *Biophys. J.* 85: 2333–2341.
*=equal contribution

IV Alakoskela*, J.-M. I., T. Söderlund*, J. M. Holopainen and P. K. J. Kinnunen, 2004. Dipole Potential and Headgroup Spacing Are Determinants for the Membrane Partitioning of Pregnanolone. *Mol. Pharmacol.* 66: 161–168.
*=equal contribution

Publication III has previously been used as a part of M.D./PhD. Tim Söderlund's dissertation "Membrane Interactions of Small Solutes – Studies with Drugs and Osmolytes", 2003, Medical Faculty, University of Helsinki.

ABSTRACT

Introduction. The lipid/water interface is important in interactions of lipid bilayers with foreign compounds as well as for interactions between lipids themselves and proteins embedded in or adsorbing to lipid bilayers. It is a region where the physical properties show drastic changes with a high level of anisotropy. These properties are also highly interconnected, and the properties of the interfacial region are known to affect functioning of proteins. Changes in the interfacial region properties have been implied in a variety of processes, such as anaesthesia.

Goals. In this work we aim at elucidating the roles of different interactions at the lipid/water interface. First, we wanted to evaluate the effect of dipole potential on interactions of membranes with small molecules and ions. Second, we hoped to elucidate other interfacial factors affecting the binding of drugs to membranes. Third, we wanted to utilize new fluorescence applications to study changes in the lipid/water interface during main phase transition. Fourth, we aimed at finding out if organic kosmotropic compounds display compound-specific effects on surface tension at the air/water interface, what the mode of action is, and if these effects correlate with those seen in lipid bilayers.

Methods. In this work we have utilized fluorescence spectroscopy accompanied with some supporting methods such as surface tension measurements to address different aspects of interfacial properties. Probes utilized include NBD, DPH, Prodan, Laurdan, and di-8-ANEPPS.

Results and conclusions. The dipole potential affects the rate of ionic reactions at the lipid/water interface. Complex changes in NBD fluorescence near transition are seen. Pregnanolone associates to the lipid/water interface, and decreases the dipole potential. Cholesterol and dipole potential-modulating compounds affect the monolayer association of pregnanolone according to Le Chatelier's principle. Thus, headgroup spacing or essentially interfacial free volume, and the dipole potential are of importance to membrane association of pregnanolone and likely for other small compounds favouring the interface. Our results show that at equal osmotic pressure sucrose, betaine, and choline change the water surface tension to a different extent. This suggests that solute-specific effects of kosmotropes at the air/water interface derive largely from the extent of preferential solvation by bulk water rather than their effect on bulk water activity. In accordance to this scheme, their effects on lipid bilayers correlate well with their effects on surface tension.

FOREWORD

The study of phospholipid membranes is a highly multidisciplinary field. There hardly is a typical background for a membrane scientist, as the background of a membrane scientist may equally well be in physics, chemistry and biological sciences, including medicine. Accordingly, a wide range of methods and approaches are used, and the problems addressed by these approaches vary greatly – from those of theoretical physics to those of medicine. The main tools used in this work are fluorescence spectroscopy and surface tension measurements, and the purpose of this text is to elucidate the role of the lipid/water interface in various membrane processes. For these reasons, in order to make this work accessible even to the beginning membrane scientists of all backgrounds, I have included a brief, simplified introduction to several of the basic aspects of both membranes and the methods used.

PART I: REVIEW OF LITERATURE

Lipids are a particularly interesting group of molecules, for they serve multiple purposes both in living organisms and in material applications. Lipid vesicles are a means of transport and delivery for both cells and for drug targeting schemes (Torchilin, 2005). Lipid membranes are semi-permeable barriers serving as the matrix of cell membranes (see Gennis, 1989) as well as in sensor applications (Toko, 1998; 2000). Lipids and lipid derivatives are used as surfactants and detergents both for physiological purposes and for human activities (Zasadzinski et al., 2001; Shivanna and Rowe, 1997). Lipid membranes are moulded into different shapes by cells, seemingly for functional purposes (Landh, 1995; Deng et al., 1999; 2002), and different spontaneously forming lipid structures have served as templates of polymer nanostructures (see e.g. Hotz and Meier, 1998). Of considerable interest is the lipid phase behaviour, particularly the lateral phase separation in cell membranes, known for over 20 years (see e.g. Pessin and Glaser, 1980; Thompson and Tillack, 1985), but only recently a matter of widespread intensive study (see e.g. Epand, 2003). It has become clear that lipid biophysics is involved in a number of cell processes, and understanding lipid phase behaviour as well as elucidating the physical properties of lipids provides a basis for the understanding of the role of lipids in cells. Phospholipids, which constitute the basic fabric of cellular membranes, are liquid crystals, and characteristically for this class of materials, they exhibit a great variety of different phases and connecting transitions (Kinnunen and Laggner, 1991). In the following I will introduce the reader to those properties and aspects of lipids and the lipid phase behaviour that provide the background for my thesis work described in the latter part of this book.

1. Structure and structural changes of lipid assemblies

The most common biological lipids one encounters in studies are glycerol-backbone lipids, sphingosine-backbone lipids and lipids based on the steroid ring structure. Among the glycerol-backbone lipids are the glycerophospholipids, and mono-, di- and triacylglycerols and their derivatives. For the phospholipids glycerol serves as the backbone into which the phosphate-containing headgroup is attached via an ester linkage and one to two alkyl chains are attached by ester or ether linkages. For the sphingolipids the backbone is formed by lysoceramide which contains already one alkyl chain, an amino group, and a hydroxyl group, to which a choline may be attached to form sphingosine. The amino group provides a possibility for amide linkage to connect a second alkyl chain to form sphingomyelin. Ceramides lacking the choline headgroup of sphingosine have a bare hydroxyl group. As the sphingolipids have not been used in the work presented here, I will concentrate on the glycerol-backbone phospholipids in the following.

The most important characteristic of phospholipids can clearly be seen simply by looking at the molecular structure: it consists of a polar part and an apolar part. More importantly, the polar part is hydrophilic in nature whereas the apolar part is hydrophobic. Having two connected parts, one easily soluble in water, the other only sparingly, is the property of amphiphilicity that together with geometrical constraints can

be used to explain several features of phospholipid behaviour. For the phospholipids there is a diversity of alkyl chains and headgroups, allowing the size and nature of the hydrophilic and hydrophobic parts to be adjusted (Gennis, 1989). The hydrophobic part makes individual phospholipids poorly soluble in water, and it drives aggregation. In water the result of free energy optimization for phospholipids is usually a structure in which the alkyl chain tails are brought together to avoid contact with water, whereas the headgroups are still facing water. Various factors such as the phospholipid headgroup and the alkyl chains, salt concentration, pH, phospholipid concentration (Cevc, 1986; 1991; Israelachvili, 1991) can all affect the effective phospholipid shape or the phospholipid/water ratio, and thus the geometry of the structure by which the alkyl chains become best removed from water (Kinnunen, 1996). Different effective molecular shapes can lead to different aggregate shapes (see Fig. I.1.1.) or phases. Different molecular shapes may also lead to stresses in existing shapes, for instance a bilayer is best formed by cylinder-like lipids but is also formed by very slightly conical lipids. Yet in this latter case the structure is stressed as the shape of the molecular building blocks and the shape required to form the supramolecular aggregate do not quite match. The different

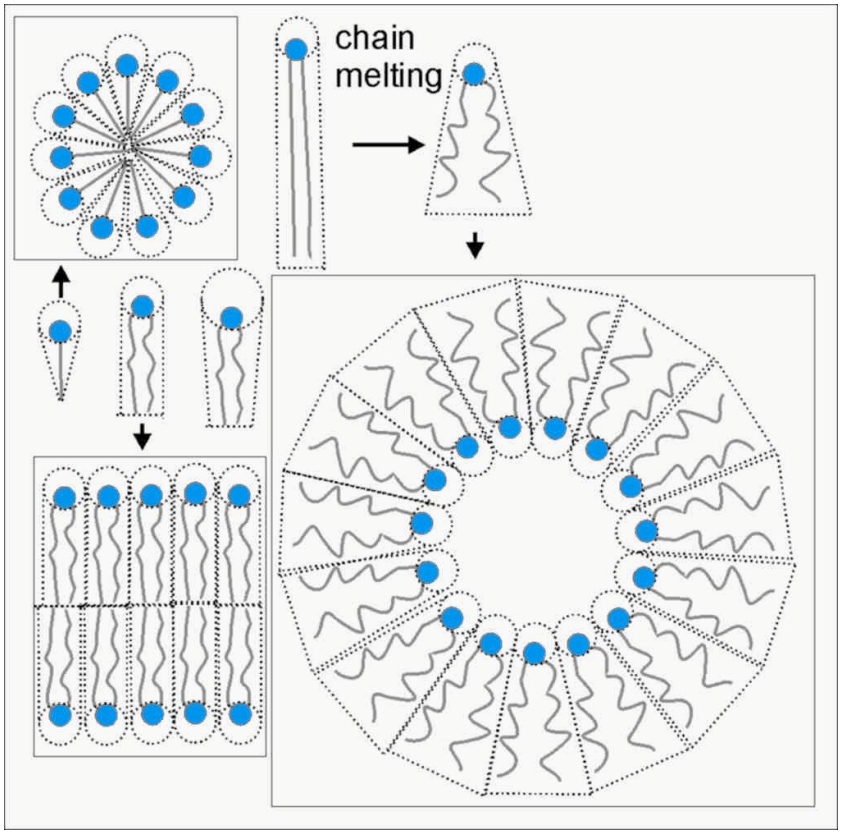

Figure I.1.1. The shapes of molecules and supramolecular aggregates. The balance between the volume under projection of the effective headgroup area, and the alkyl chain volume is one of the main determinants. The effective headgroup area itself is affected e.g. by hydration and pH, and the shape obtained by alkyl may also be of importance (Cevc, 1986; Israelachvili, 1991; Kinnunen 1996).

nonbilayer phases and transitions connecting them to each other or to bilayer phases (Tate et al., 1991) are of interest for some applications (Caffrey, 2003; Lee et al., 1995; Yang et al., 1996), and possibly biologically relevant (Landh, 1995; Deng et al., 1999; 2002; Kinnunen and Holopainen, 2000). However, biologically the most important phases are undoubtedly bilayer phases, and as only bilayer phases are of importance for the work presented here, the discussion henceforth will be restricted to them.

1.1. Bilayers

The discovery of the bilayer nature of biological membranes traces back to the experiments of Gorter and Grendel (1925). Despite their flawed experimental design by comparing monolayers of red blood cell lipid extracts to the area of the red blood cells they ended up with the correct conclusion that the plasma membrane of red blood cells is formed by two layers of lipids with their acyl chains directed towards the centre of the bilayer, similarly to amphiphile bilayers previously described. Indeed, this allows the hydrophobic alkyl chains to be removed from water, and the hydrophobic rim can be removed by curving the bilayer sheet into a vesicle. This curving into a closed vesicle introduces constraints related to the curvature energy and thus the size of the vesicle affects phase behaviour (see e.g. Lichtenberg et al., 1984). Basically, for smaller vesicles a local curvature change causes severe balancing distortions elsewhere in the vesicle. As for larger vesicles with excess area these balancing distortions may be evened out over large membrane areas, and curvature properties are due to those of bilayer sheets. The structure of bilayer sheets have been widely studied by x-ray and neutron diffraction techniques (Nagle and Tristram-Nagle, 2000; Wiener et al., 1991; Wiener and White, 1992a,b). For such structural studies they are in many ways ideal, but not perfect. X-ray techniques provide excellent data about the solid-like phases, but are less exact for fluid phases (Nagle and Tristram-Nagle, 2000). The measurement and data analysis are often too costly in terms of time and money for the studies of dynamics and changes in bilayer properties. This is the realm where fluorescence spectroscopy flourishes, as it allows the use of probes to test changes in bilayer properties from simple, inexpensive, fast experiments. Importantly, while gross structures of different bilayer phases are similar, their differences are of utmost importance for their properties, as evidenced by the fact that transition from fluid-like to gel-like bilayer phase in cell membranes is believed to be the cause of death for rapidly dehydrated *E. coli* (Beney et al., 2004). A brief summary of the properties of different bilayer phases follows.

1.2. Lipid phases

For pure bilayer-forming phospholipids, especially phosphocholines, one typically sees a sequence of bilayer phases with increasing temperature: $L_c/L_{c'}$, $L_\beta/L_{\beta'}$, possibly $P_{\beta'}$, and L_α. The apostrophe (') in the subscript of a phase symbol indicates tilted alkyl chains in this phase. The lowest temperature L_c phase is a crystalline-like phase in which the alkyl chains are all-*trans* configuration and the headgroups also form a well-ordered lattice with a unit cell of two phospholipids (Raghunathan and Katsaras, 1995). For the gel-like $L_{\beta'}$ phase, which will be referred to as the gel phase (see Fig. I.1.3.), the alkyl chains remain in an all-trans configuration while the headgroup dipole lattice becomes

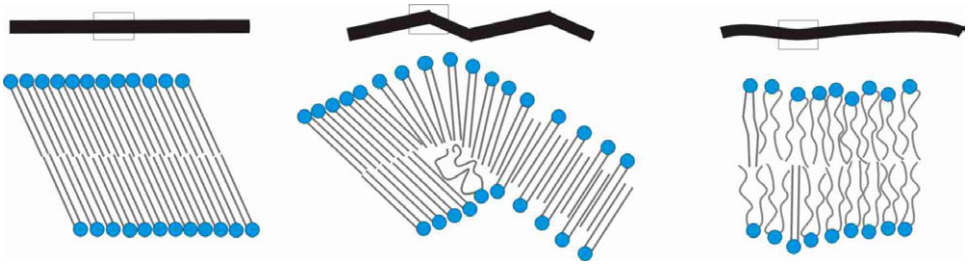

Figure I.1.3. A schematic illustration of some phospholipid phases. The order of phases from left to right is $L_{\beta'}$, $P_{\beta'}$, and L_{α}. The upper bar shows a cross-section of membrane. The lower image shows a more detailed scheme of the ordering of acyl chains. In the $L_{\beta'}$ phase the cross-section is straight, and the acyl chains are highly ordered. In $P_{\beta'}$ phase there is sawtooth like periodicity with 14.2 nm periodicity for DMPC, and with the longer segments being thicker than the shorter segments (Nagle and Tristram-Nagle, 2000). Until recently the distribution of *gauche* bonds within the $P_{\beta'}$ phase was unknown. A recent atomistic molecular dynamics simulation reproduced the experimental periodicity and thickness of the ripple phase. The thinner segment was identified as an interdigitated phase (Vries, de, et al., 2005). In the L_{α} phase there are thermally excited undulations and protrusion of lipid molecules, the disorder of acyl chains is discussed in more detail in the text. Modified from Alakoskela and Kinnunen, 2004 based on de Vries et al., 2005.

Table I.1. Some characteristics of phosphocholine phases

Property and lipid	In $L_{\beta'}$ phase	In $P_{\beta'}$ phase	In L_{α} phase
rate constant for CF permeability in DPPC matrix[5], 10^{-4}/s	≈ 0.1–0.2 (20°C)	≈ 2 (37°C)	≈ 3–4 (50°C)
area / lipid, Å^2			
DPPC[1]	47.9	–	64
hydrophobic thickess, Å			
DPPC[1]	34.4	–	28.5
volume / lipid, Å^3	(20°C)		(50°C)
DPPC[1]	1142–1145	–	1228–1232
thermal area expansivity, K^{-1}			
DMPC[2]	3	5800	6800
elastic area compressibility modulus, mN/m			
DMPC[2]	860	62	145 (at T*)
DMPC[6]	526	500	476 (T-T_m=11ºC)
fraction of *gauche* bonds			
compiled data in 1991[3]	0–0.1	–	0.3
MD with corrections[4]	–	–	0.28
MD without corrections[4]	–	–	0.22
derived from IR spectra[4]	–	–	0.14±0.04

[1]Nagle and Tristram-Nagle, 2000. [2]Needham and Evans, 1988, direct mechanical measurement.
[3]Marsh, 1991. [4]Snyder et al., 2002.
[5]Bramhall et al., 1987. [6]Lemmich et al., 1996, based on calculations from neutron scattering data. Please notice that these values do not imply the existence of a net tension. Instead, tension is balanced by pressure. Modified based on Alakoskela and Kinnunen, 2004.

disordered. As lateral diffusion in $L_{c'}$ and $L_{\beta'}$ phases is exceedingly slow (Raghunathan and Katsaras, 1995), so is the reordering related to transitions between them. As the temperature increases, one sees for the phosphocholines and for some other phospholipids a transition to the $P_{\beta'}$ phase, which is the rippled gel-like phase. This transition is called the pretransition, as it is soon followed by a transition into a fluid-like, liquid-crystalline L_{α} phase, which will be referred to as the fluid phase. In the $P_{\beta'}$ phase

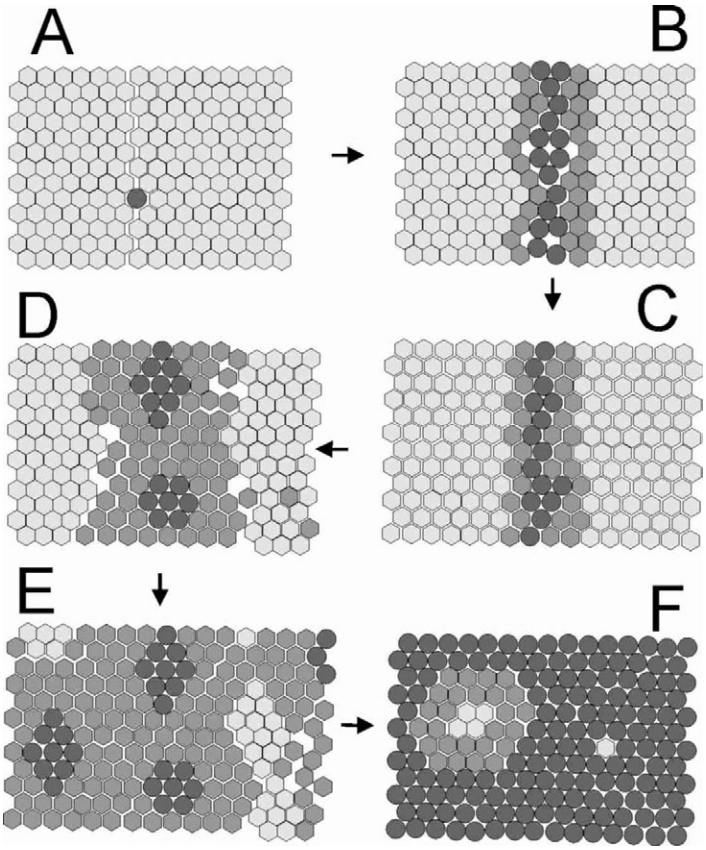

Figure I.1.4. A scheme of changes in the vicinity of the main phase transition. Panel A shows $L_{\beta'}$ phase with a single excited lipid with disordered acyl chains. The larger area required by the excited lipid imposes strain on the $L_{\beta'}$ phase lattice, resulting in a fault line. This strain is relieved by packing the excited lipids into line defects (panel B), which leads to the formation of the $P_{\beta'}$ phase (Heimburg, 2000; see also Fig. 1). Along the line defects there should exist lipids with an intermediate order or boundary-lipid like nature. With further increase in the temperature the lattice becomes more loose, yet without sudden increase in area (such a sudden increase not taking place in reality is shown in panel C). Rather this allows for the fluid-like domains to become dispersed into the bulk from the line defects, and new fluid phase domains to form (and disappear) due to phase fluctuations (panel D). The domains are likely to be surrounded by layers of boundary lipids, giving the whole bilayer a boundary-like nature due to the large fraction of boundary lipids (panel E). Gradually the fraction of fluid phase increases, leaving only gel-phase domain heterofluctuations and single all-*trans* phospholipids in a fluid matrix (panel F). With further increase in temperature the fluctuations will cease and the fraction of all-*trans* lipids approaches zero. Modified from Alakoskela and Kinnunen (2004).

the degree of alkyl chain *trans→gauche* isomerization is intermediate between that of the $L_{\beta'}$ and L_{α} phases. The L_{α} is a fluid phase, with considerable fraction of *trans→gauche* isomerization and relatively fast lateral diffusion. The existence of the $P_{\beta'}$ phase appears to be dependent on the lipid headgroup, as the $P_{\beta'}$ phase is absent for phospholipids with phosphoethanolamine headgroup (Cametti et al., 1991). This suggests that tilting or interdigitation that is likely to be present due to larger headgroup of phosphocholines, phosphoglycerols, and phosphoserines is necessary for the existence of the $P_{\beta'}$ phase. While the level of *trans→gauche* isomerization is known to be intermediate between the gel and fluid phase, the exact spatial distribution of this chain disorder in the ripples is uncertain (Nagle and Tristram-Nagle, 2000). The transition from either $P_{\beta'}$ or directly from L_{β} phase into L_{α} is called the main phase transition. In the schematic illustration of figure I.1.3 some typical features are shown, and some properties of some of the phases are summarized in Table I.1. Not only are the properties listed in Table I.1. different in different phases, but the changes in transitions from one phase to another are abrupt.

1.3. Phase transitions

Despite considerable effort taken over the last century phase transitions remain a fundamental problem in physics. For long it was believed that three phases for matter existed – solid, liquid, and gas – and that consequently only one melting transition existed. The class of materials that came to be known as liquid crystals emerged in 1888, when Friedrich Reinitzer discovered two melting transitions for cholesterol benzoate (see Prost and Williams, 1999). Phospholipids, that are liquid crystalline matter, similarly exhibit several phase transitions between phases, for example the gel-like and liquid-like lamellar phases described above.

While the nucleation phenomena and the sensitivity of predictions to the exact accuracy of intermolecular potentials have prevented the generation of a general, predictive theory of phase transitions (Papon et al., 2002), multiple slightly differing phenomenological models and models accounting for the thermodynamics of phase transitions have appeared, and the basis of the phospholipid main phase transition is well known. The driving force of the phospholipid main phase transition derives from the configurational entropy of the acyl chains, i.e. *trans→gauche* isomerization. For the $L_{\beta'}$ phase the fraction of *gauche* bonds is most likely close to zero, while for the L_{α} phase values ranging between 0.14–0.3 have been suggested (Marsh, 1991; Snyder et al., 2002; see Table I.1), and the probability of finding a gauche bond increases toward the centre of the bilayer, from approximately 0.1 in first two bonds vicinal to the glycerol backbone to approximately 0.3 at their methylene ends. In this work as well as frequently in literature the increase and decrease in the fraction of *gauche* bonds are referred to as decrease and increase in chain order, respectively.

As the fraction of *gauche* bonds abruptly increases during the phase transition, the alkyl chains become kinkier, and the thickness of the bilayer decreases by approx. 16 %, and the area increases by approx. 25 % (see Heimburg, 1998). However, kinks in the chains also increase the amount of voids and water penetration into the bilayer. Therefore the volume of the bilayer increases by approx. 4 % (see Heimburg, 1998). Accordingly, both area and volume of the bilayer are coupled to the order of the alkyl chains. The

transition enthalpy is similarly coupled to the chain order. Even for different phospholipids there exists a general constant coupling enthalpy (ΔH) and volume changes (ΔV), and ΔH curve is identical in shape to ΔV curve, and heat capacity (C_p) curve is identical in shape to volume isothermal compressibility (κ_T) curve (Ebel et al., 2001; Halstenberg et al., 1998; Heimburg, 1998; Schrader et al., 2002).

The series of phases illustrated I.1.3., $L_{\beta'}$, $P_{\beta'}$ and L_α are connected by pre- and main transitions. Heimburg (2000) has suggested that the pretransition and the associated structural change of rippling results from line defects formed by phospholipids with melted acyl chains. Following this, melted and unmelted lipids should segregate to surfaces of different curvature within the ripples. Although the model contains some features which are not completely in accordance with the novel MD simulation (de Vries et al., 2005), Heimburg's model does reproduce experimental enthalpy changes for the transition of both unilamellar and multilamellar vesicles (MLVs) reasonably well, illustrating the nature of coupling between the pre- and main transition. While the pretransition is more prominent in the case of multilamellar vesicles due to high co-operativity of rippling, it is observed also for unilamellar vesicles although the enthalpy associated to the pretransition of unilamellar vesicles is much smaller than that of MLVs and the detection of ripple structure is not as easy (Heimburg, 1998; Lichtenberg et al., 1984; Mason et al., 1999). Unilamellar vesicles(giant vesicles excluded) are typically smaller than MLVs and more free to deform, especially if there is excess surface area compared to volume. In regard to the line defects in large unilamellar vesicles, it is noteworthy that while the unilamellar phospholipid vesicles above the main phase transition are almost smooth spheres, the vesicles below the main phase transition temperature T_m are only roughly spherical. They are composed of irregular, straight facets or plates joined by fault lines, as seen in cryo-TEM images (Andersson et al., 1995; Schneiner et al., 1999). In unilamellar vesicles these defect lines could be the place of initial melting and the first location of line defects.

The properties of the phospholipid bilayers show discontinuities at the main transition, as expected (see Table I.1). In addition to differences between phases, some properties, for example membrane permeability, heat capacity and the lateral compressibility (Langner and Hui, 1993; Mouritsen and Kinnunen, 1996; Nagle and Scott, 1978) have maxima at T_m. These as well as other findings have been explained in terms of dominating critical density fluctuations leading to the formation of domains of gel- and fluid-state lipids, and further to maxima in the domain boundary length and the number of defects in membranes. The above, however, is in conflict with fluorescence spectroscopic results, which suggest that this model might not be sufficient, and further imply that the fraction of lipids in the phase boundary is high (Jutila and Kinnunen, 1997; Metso et al., 2003). These aspects have been recently reviewed, and are presented in Fig. I.1.4. (Alakoskela and Kinnunen, 2004). In theoretical models of the main phase transition all but the acyl chain degrees of freedom are assumed constant, which neglects the mobility of phospholipid headgroups as well as interfacial water (for a review see Mouritsen, 1991). Indeed, such simplifications are justified, as present theories reproduce most of the essential features to a great detail. In recent models a first-order (or a weakly first-order) nature is usually assigned to the phospholipid main phase transition. Yet, the transition is quite wide; the increase in enthalpy is steep but not stepwise (Mouritsen, 1991). Phospholipid membranes also display extensive fluctuations several degrees away from the transition (see Kharakoz and Shlyapnikova, 2000, and references therein), which

would be typical for second-order transitions. Due to these observations, a suggestion of a weakly first-order nature due to the presence of a critical point close to the transition (Mouritsen, 1991; Lemmich et al., 1995) as well as an alternative suggestion of extensive heterophase fluctuations (due to low line-tension between fluid-solid interface) (Kharakoz and Shlyapnikova, 2000) has been put forward to explain the pseudocritical nature of the transition. The phase behaviour of lipids is greatly affected by changes in the lipid-water interface, and by molecules or other particles associating to the lipid-water interface (Boggs, 1987; Cevc, 1986; 1991). However, these effects do not affect the nature of the transition in the sense that the transition is and remains as a collective change of acyl chain order and possible effects in the interface appear to be secondary to the acyl chain behaviour. However, in the pursuit of the molecular level details of how the transition proceeds, it is of interest to take a look at the changes in the lipid-water interfacial region as well. Interestingly, the polar headgroup and the interfacial water organization have been suggested to show discontinuities slightly below T_m, as found in fluorescence (Jutila and Kinnunen, 1997, Söderlund et al., 1999), IR (Mellier and Diaf, 1988; Mellier et al., 1993), and dielectric spectroscopy (Enders and Nimtz, 1984) studies on DMPC and DPPC liposomes. Likewise, a new kind of critical behaviour is observed above T_m as well, at the so called critical unilamellar temperature, T*, at which the last gel-like nuclei disappear upon heating (Gershfeld, 1989a; 1989b). Several discontinuities in membrane properties occur at this temperature, and these changes appear to be connected also to membrane hydration (Gershfeld and Ginsberg, 1997; Gershfeld et al., 1993; Jin et al., 1999; Koshinuma et al., 1999; Lehtonen and Kinnunen, 1994).

2. Role of lipid variety in biological membranes

One of the great unanswered questions of membrane research is the reason for the very large amount of lipid species in the cell membranes. For some of the species specific roles as second messengers, substrates for messenger molecules (see e.g. Alberts et al., 2004) or for very specific physical functions typical of single cell type as the water impermeability obtained largely by skin ceramides can be found. However, a vast majority of the cellular membrane lipids do not fall into these categories. As already discussed, achieving phase separation could account for another small part of the variation. A likely possibility is that the rest are involved in regulation of a variety of physical properties of membranes or those of different membrane domains separately, including such properties as phase behaviour, electrostatics, different types of permeability barriers, and possibly even distribution of lateral pressures along the membranes.

In some simple, well-defined cases much is known. For example, the transversal asymmetry in distribution of anionic phosphatidylserine is actively maintained (see Balasubramanian and Schroit, 2003), most likely for achieving electrostatic interactions with proteins in the inner leaflet of membrane while avoiding the reactive, charged surfaces on the outside of cells. Similarly, to mention a few examples, it appears that a propensity for inverted nonlamellar phases, specifically the H_{II} phase is maintained. The composition of cell membranes is such that the membrane lipids typically undergo transition to H_{II} approx. 20°C above the growth temperature of the cell (Kinnunen, 1996). Moreover, it appears clear that this adjustment of the physical properties of the

membranes is the primary goal of this regulation and not secondary to the regulation of the mole fractions of lipid species. For instance in *Acheloplasma laidlawii*, an organism dependent on an outside source for acyl chains, the fractions of lipid headgroups are altered to produce the same propensity for H_{II} phase if the nature of acyl chains is varied. Another example is that a foreign phospholipid with similar physical properties can substitute for a native phospholipid (Karlsson et al., 1996; Vikström et al., 1999; Wikström et al., 2004). Although it is difficult to pinpoint the exact meaning of this finding of the H_{II} propensity regulation in the complex environment of cell membranes, some possible reasons could be due to the decrease of the energy barriers for the budding and the fusion of vesicles (Deserno, 2004), the regulation of the energy barrier for the extended conformation of lipids (Kinnunen, 1996), and the adjustment of lateral pressure profile (see later). Directly related to the phase behaviour is the modification of the cell membrane composition by cells in order to keep the critical unilamellar temperature of the membrane equal to the growth temperature (Ginsberg et al., 1991; Jin et al., 1999). As discussed already, this temperature apparently corresponds to the disappearance of the last gel phase nuclei in the bilayer consisting purely of the lipids of the cell membrane, and its name is due to the spontaneous formation of bilayers at air/water interface at this temperature (Gershfeld, 1989a; 1989b; Gershfeld and Ginsberg, 1997; Gershfeld et al., 1993; Koshinuma et al., 1999). The exact reason for keeping the plasma membranes at T* is not known.

While the above provides a few simple parameters shown to be targets of either direct or indirect regulation by cells, it is quite likely that other, less easily determinable physical properties are also targets for regulation by cells. What makes this notion more than a fancy is that many properties of lipid bilayers have been shown to affect the functions of both integral and peripheral membrane proteins (see de Kruijff, 2004), and thus it would be logical that they, too, would be regulated by cells. Among such properties, to mention a few, are the membrane dipole potential, the hydrophobic length and the lateral pressure profile.

3. Hydration and water

Water is the most abundant molecule in living organisms both by weight and by numbers. Water is also one of the most surprising small molecules, and it provides perhaps the most striking example of difficulty in reproducing properties of liquids even with the best simulation techniques available. A comprehensive summary of the many surprising properties of water can be found at Prof. Martin Chaplin's web pages "Water: the structure and behavior" (http://www.lsbu.ac.uk/water/) with their over 700 references.

For the purpose of an introduction for this study, we can barely scratch the surface of the wealth of interesting behaviour and interactions of water. Obviously, for this purpose the most important features are connected to the interfacial hydration, and to the surface and interfacial tension of water and its modifications by kosmotropes and chaotropes. To elucidate the interconnectedness (and lack of it) of phenomena, a brief introduction into the relationship between osmotic pressure and change in surface tension is necessary.

3.1. The structure and properties of water in bulk and near interfaces

The structure of liquid water remains a matter of active debate and study. Evidently, hydrogen bonded networks are present in water (Head-Gordon and Hura, 2002), and suggestions of different type of more elementary hydrogen-bonded oligowaters (see Chaplin, 1999) or different states of water (Urquidi et al., 1999; Sciortino et al., 2003; Dill et al., 2005) have been made. One important aspect of interwater hydrogen bonding is the polarizability of water, which leads to co-operativity of hydrogen bonding (Luck, 1998). Indeed, the dipole moment of a water molecule in bulk water is quite different from that in gas phase, the former being approx. 2.6–3.0 D and the latter 1.85 D (Head-Gordon and Hura, 2002). Additionally, to fully explain the dynamics and spatial distribution of room temperature water one needs to take into account the true quantum effects like uncertainty in orientation and tunnelling (Hernández de la Pena and Kusalik, 2005). On the other hand, a simple model (called Mercedes-Benz model because the shape of water molecule in model is similar to MB logo) of two-dimensional water as circular disks with three points of attachment reproduces qualitatively many observations e.g. dissimilar behaviour of small and large hydrophobic solutes (Dill et al., 2005). While hydrogen bonding networks likely play the most important role in properties such as a maximum in the density of liquid water at 3.984°C, a heat capacity maximum at approx. 36°C (Chaplin's web page http://www.lsbu.ac.uk/water/; Dougherty and Howard, 1998) and an ability of water to dissolve well not only polar but also apolar compounds among other things, the polarity of water molecules is important e.g. for the large relative permittivity of water. Most of the large static permittivity of water originates from the reorientation of water molecules, as is demonstrated by the fact that the relative permittivity at optical frequencies is considerably smaller than static permittivity. Nevertheless, water very effectively decreases the strength of electrostatic interactions, and, thanks to its ability to dissolve large amounts of most salts, a saline solution with a far greater effect in this respect can be prepared.

One of the important features is that solutes appear to have solute-specific interactions by mechanisms which have been a matter of intensive study and are not included in the simple theories or models commonly used. Some aspects of this behaviour are discussed in the next section. Interestingly, also dissolved gas affects interactions in water to a great extent (see e.g. comprehensive review by Čolić and Miller, 2000). One aspect of this phenomenon is that there is a strong, relatively long-range attractive force between hydrophobic surfaces in water and this force becomes stronger with dissolved gas. Among the explanations to account for this phenomenon are the presence of pre-existing nanobubbles in water, the enhancement of statistical density fluctuations and cavitation in the presence of gas, leading to a bridging between surfaces (see Čolić and Miller, 2000). New experiments appear to support the last alternative, giving reduced water density over a region of 1.13 nm and a range of approx. 25 nm for long-range attraction thus agreeing with theoretical predictions (Doshi et al., 2005). Evidently the results of most biochemical and biophysical measurements made in water are dependent on the interactions between the studied particles, and a simple degassing may affect the results drastically.

The gas effect discussed is connected to the exclusion of water from regions near the interface of a hydrophobic particle. One closely connected aspect of this diminished density of water is the interfacial or surface tension. Basically, density depletion of water is due to the more favourable interactions of water with the bulk water than with the

particle or substance on the other side of interface, and for this reason the density of water near the interface is lower, following a Boltzmann distribution. The surface tension of water is particularly high, and it results from the strongly polar and hydrogen-bonding character of water. Only recently a more complete picture has begun to arise. For example the fraction of water molecules participating in four hydrogen bonds is 23 % in bulk and only 4 % on the surface as seen in an *ab initio* MD simulation (Kuo and Mundy, 2004), in keeping with experimental observation. The thickness of the density-depleted surface water layer beneath the surface (equalling the height at which density is 50 % of that of bulk water) is approx. 1 nm (Kuo and Mundy, 2004), interestingly this coincides with the critical length scale between small and large hydrophobic solutes (Huang and Chandler, 2000). Expectedly, the properties of interfacial water are quite different from those of bulk water. The relative permittivity of water changes from a value of 4 close to mica surfaces to the bulk value of 80 within a 10 nm distance (Teschke et al., 2001a,b), and likely similar behaviour is found near the surfaces of biological molecules (Despa et al., 2004). There is an interesting discrepancy in this: the 10 nm layer of the permittivity gradient is much larger than the 1 nm layer of density gradient. Speculatively, a restricted orientational relaxation could explain the permittivity gradient beyond 1 nm, but this would nevertheless be somewhat surprising. In very thin water films of only a few molecules thick the effective viscosity of water between mica sheets is much higher than bulk viscosity (Zhu and Granick, 2001), but probably this alone cannot explain this deviation, especially since despite the lower hydrogen bonding dynamics of surface water the molecules just beneath these show faster dynamics (Paul and Chandra, 2004). Another feature of interest is that the molecular orbitals of surface water are arranged differently from those in bulk, making the surface reactive to excess protons and electrons (Kuo and Mundy, 2004). These features, together with polarizability, surface dipole potential and size effects, are likely to explain the preferential partition of some ions into the interface at low concentrations. The most important of these properties are polarizability and solvation energy, i.e. the hydration of the ion, both of which include many size effects. For example, polarizable ions, typically larger anions, such as chloride, tend to accumulate at interfaces (Petersen et al., 2004; Jungwirth and Tobias, 2002; Boström et al., 2005).

3.2. Solute effects on surface tension and its relation to osmotic pressure

Thermodynamics of solute effects on surface tension are well known and easily explained in terms of the Gibbs adsorption isotherm. Basically, if a solute favours the interface over the bulk, it adsorbs to the interface, and has a positive excess surface concentration. The free energy gain of adsorption contributes to the lowering of the surface tension of the solution. On the other hand, if the solute has more favourable interactions in bulk compared to surface, it is depleted from surface region, and has a negative excess surface concentration (see e.g. Butt et al., 2003). In this, the surface tension increases with increasing solute concentration. However, it is often not easy to know whether the forces are favourable or unfavourable for adsorption or surface layer partitioning. Additionally, the thickness of interface and available interfacial sites has an effect. As an example of complexity of surface tension effects let us take a look at simple salts. Simplified theory by Onsager and Samaras (1934) treats ions as point charges in continuum dielectric solvent, and suggests that not only should the surface tension of solution increase

continuously with increasing salt concentration but that all 1:1 salts should have an equal effect. Their theory covers only the electrostatics of the image forces and fails in more than one respect to correspond to reality, while likely providing a relatively good approximation for intermediate salt concentrations of approx. 5–100 mM. The failure of Onsager–Samaras theory at low concentrations was first discovered as early as 1937. Below the concentration of 1 mM a wide variety of salts actually decreases surface tension (Jones and Ray, 1937; 1941). At the time this was apparently correctly explained in terms of preferential partition of ions into the interface until the interface is saturated (Dole, 1938; Dole and Swartout, 1940), although later studies indicate that the preference of OH^- to the interface is of importance as well (Karraker and Radke, 2002). Incidentally, Langmuir (1938) suggested this effect to derive merely from a systematic error of capillary rise method, based on theoretical arguments. However, recently theories less simplified than the Onsager-Samaras (Manciu and Ruckenstein, 2003), simulations incorporating polarizability (Jungwirth and Tobias, 2002) as well as the direct experimental verification of surface enrichment of ions (Petersen et al., 2004) have proved the accuracy of early observations. Possibly the special properties of surface water contribute to this behaviour (see previous paragraph), or, alternatively, bulk water may push out ions fitting poorly into bulk water structure. The Onsager-Samaras theory fails also in the respect that at higher concentrations different 1:1 salts have very different effects on surface tension, a fact proved by experiments whose results are reproduced by a novel theory incorporating both the solvation energies and the (frequency dependent) polarizabilities of ions (Boström et al., 2005). Of course, an increase in surface tension despite the (initial) enrichment of ions into the surface layer still obeys a Gibbs adsorption isotherm when one takes into account that the thickness of the interface changes, contributing significantly to energies. One just has to dispense the notion of a simple, continuous decay of ion concentrations. As discussed by Butt et al. (2003), very different surface density depletion profiles may result in the same observed surface excess concentration and surface tension.

Notably, these surface tension effects are closely connected to the Hofmeister series (Boström et al., 2005; see also Kunz et al., 2004b) and the concepts of chaotropic and kosmotropic solutes (see particularly Manciu and Ruckenstein, 2003). Specific ion effects

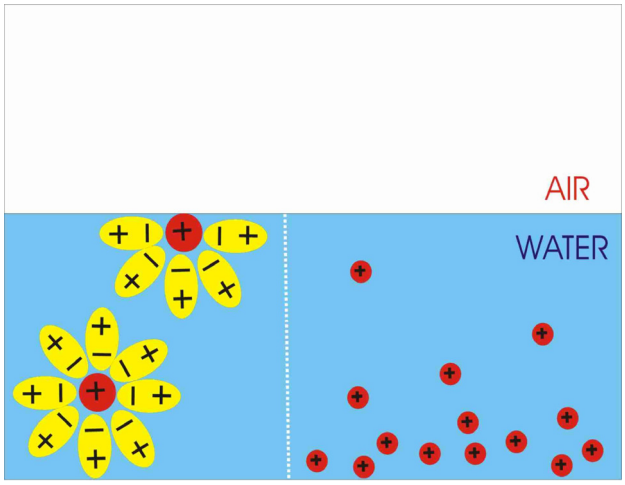

Figure I.3.1. A simplified view of the basis of the effect of ions on surface tension. Hydrating water molecules have to be removed for an ion to reach surface (left). For an ion slightly farther away from interface the secondary water molecules reoriented by the hydration layer are removed. It is thus energetically unfavourable for the ions to partition to the immediate vicinity of the interface. Thus ions distribute according to Boltzmann distribution (sketch on right). This makes the energetics even more unfavourable for interfacial water, leading to an increase in surface tension.

are ubiquitous in colloid science and biochemistry. Often these effects follow this so-called Hofmeister series, which can be accounted by including the ion solvation energies and the polarizabilities to the models (Boström et al., 2005; Manciu and Ruckenstein, 2003). An alternative qualitative description of the series describes it solely in terms of the ability of ions to enhance or perturb the structure of liquid water. The term chaotrope is assigned to an ion species with supposedly strong perturbation of water structure, and the name kosmotrope is assigned to an ion species with supposedly strong structure-enhancing effect. As discussed by Chaplin (web review, see also review in 1999), many of the effects can be qualitatively described simply by allowing for different hydration energies of ions. Also the solubilities of salts appear to depend strongly on the kosmo- or chaotropic nature of the ion pair, as salts consisting of two kosmotropic or two chaotropic ion species are much more soluble than salts consisting of one kosmotropic ion and one chaotropic ion species.

Not all kosmotropes and chaotropes are ions; in fact, many organic compounds used by cells as protection against osmotic stress or freezing are kosmotropes. Among these are, for example, betaine (Galinski et al., 1997) and the disaccharide trehalose (Galinski et al., 1997). Likewise, sucrose is a kosmotrope (Herberhold et al., 2004). Such solutes are often called compatible solutes, as they are preferentially excluded from protein surfaces (thus essentially increasing the water/protein surface tension or surface energy, to be more precise) and by thus stabilizing the native conformation of proteins they are compatible with life. Alternatively, they are called compensatory solutes, as they compensate for osmotic pressure outside the cell (Gilles, 1997). At this point we take a closer look at the connection between the osmotic activity and surface or interfacial tension. In a simplified view one accounts for the negative surface excess concentration at a given bulk concentration of solute by utilizing an abstraction: the system is divided into three separate zones of 1) gas or vapour, 2) interfacial liquid zone completely devoid of solute, and 3) bulk liquid zone. In this kind of model, the work done against osmotic pressure to create the surface layer 2 of a defined thickness equals the surface tension increment, and gives a connection between the Gibbs adsorption isotherm and osmotic pressure (Parsegian et al., 2000). This kind of abstraction helps to view the origins of different surface tensions caused by same concentration of added solutes. Firstly, the interactions of the solutes with bulk water may be different, leading to different osmotic pressures for same concentrations of different solutes. Secondly, the differences in the surface tension increase may be derived from different interactions of solutes with the interfacial water as compared to the bulk water. This latter scenario would correspond to a change in the thickness of the interfacial region in the simple model, and, accordingly, the surface tension increase by a solute may be different even when the osmotic pressure is the same for different solutes. One of the aims of our work was to address the modes of surface tension increase by biologically relevant organic kosmotropic solutes.

3.3. Hydration of biomolecules

Molecules in solution interact with water in a complex way, and biomolecules are no exception. Both the kinetics and the steady-state of solvation by water, i.e. hydration, of simple and biological molecules have been studied extensively, with much emphasis on the understanding the hydration of the hydrophobic compounds giving rise to a hydrophobic effect (see e.g. Israelachvili and Wennerström, 1996; Kaatze, 1997; Nandi et

al., 2000; Orozco and Luque, 2000; Parsegian et al., 2000; Dill et al., 2005). The hydrophobic effect is required for the formation of the double helix of DNA. The level of hydration controls the conformation of DNA (see e.g. Israelachvili and Wennerström, 1996; Orozco and Luque, 2000) as well as that of proteins, and it also allows for fast conformation fluctuations of proteins (Sorensen et al., 1999; Orozco and Luque, 2000; Li et al, 2001; Rowe, 2001; Timasheff, 2002). In addition, hydration affects the functioning of proteins as expected based on the "structure is function" paradigm, (Colombo et al., 1992; Barrabin et al., 1993; Giorgione and Epand, 1997; Li et al., 2001).

The level of hydration is dependent on the osmotic pressure, thus coupling the number of associated water molecules to bulk properties (see Parsegian et al., 2000; Rand et al., 2000). The interactions between molecules are also affected by changes in osmotic pressure, as the association of molecules, say receptor and ligand or two proteins, usually changes the number of water molecules associated to them, typically releasing water from associating surfaces. In addition, models predict attractive interactions between surfaces for this reason, as well as oscillatory repulsion due to release of water from space between surfaces (see Israelachvili and Wennerström, 1996). One should not make an error and believe that there is a large number of tightly associated water molecules in biomolecules such as proteins, as results clearly show that there is fast exchange. Nevertheless, the weak, short-lived association does affect the properties of this hydration water. For example viscosity (Halle and Davidovic, 2003) and, obviously, dielectric relaxation are affected due to this very same dynamically ordered layer (Kaatze, 1997; Nandi et al., 2000; Pal and Zewail, 2004).

In the case of phospholipid bilayers the hydrating water molecules are less hydrogen-bonded than those in water, and there is a considerable dynamic orientational ordering of water at the lipid/water interface, which is dependent on lipid headgroup (Cheng et al., 2003). The hydration or the ordering of water at the lipid/water interface has several levels. Depending on the headgroup, phospholipids bind approx. 0.5–3 water molecules/lipid very tightly ($E \approx 40$ kJ/mol; Jendrasiak et al., 1996). Interaction between lipids appear to be nearly the same as in excess water when approx. 10 water molecules/PC is reached, as judged by T_m and lipid rotational mobility, whereas the bilayer appears more or less saturated at 22 water molecules/PC (Ulrich and Watts, 1994). Simulation studies have shown tetrahedral ordering in the interface, and orientational ordering extending approx. 7 Å into the water phase from the average positions of the outermost phospholipid groups (see e.g. Jedlovszky and Mezei, 2001). While an extensive lattice suggested in early models (Forsling and Kjellander, 1975) does not exist, on average the choline group appears to have a clathrate like shell, and the phosphate group shows solvation more typical of ions with the first hydration shell of headgroups giving altogether 17–22 molecules (Alper et al., 1993). The hydration of (different) phospholipids has been studied extensively, and cannot be fully reviewed here. For this, and the effects of hydration on the desorption of compounds from vesicles, the phase behaviour of lipids and the dynamics of lipids the reader is referred to work addressing these features (Jendrasiak and Hasty, 1974; Taylor et al., 1977; Ter-Minassian-Saraga and Madelmont, 1982; Cevc, 1988; Shin et al., 1993; Slater et al., 1993; Ariga and Okahata, 1994; Ho et al., 1995; McIntosh, 1996; Hsieh et al., 1997; Bach and Miller, 1998; Channareddy and Janes, 1999). Yet, hydration appears in many other works. Due to the unknown bulk water structure and the long-standing inaccessibility of the interfacial water structure in experiments, hydration has often been

employed in colloid science to explain unexpected observations, as discussed by Israelachvili and Wennerström (1996).

4. Surface tension and internal pressure in lipid films

The forces that give birth to surface tension exist at interfaces between lipid films and bulk phases as well. However, an interface covered by a surface active species such as lipids does not have a real, constant surface nor interfacial tension dependent only on temperature. (Often surface tension is reserved exclusively for an interface between a liquid and its vapour, whereas interfacial tension is used for other kinds of interfaces.) Instead interfacial tension changes as a function of area, because the changes in area change the packing density of the surface active compound as well. This in turn changes the extent of shielding between the solvo-/hydrophobic and solvo-/hydrophilic phases. Thus, one obtains a different value for γ for each surfactant coverage. In the following text, interfacial tension is used to describe γ in this sense, dependent not only on temperature but also on the molecular packing density of the surface active species.

4.1. Interfacial tension in bilayers

As described above, every interface has interfacial tension. The interface between the lipid bilayer surface and water is not an exception, the interactions in bulk water are obviously different from those between interfacial water molecules and headgroups and exposed acyl chains of the lipids. Yet, the bilayer is a system free to equilibrate, and consequently this interfacial tension drives the decrease in the bilayer area until the internal pressure of the bilayer balances the interfacial tension. For bilayers the situation is very complex, since both the interfacial tension and internal pressure are dependent on the area per molecule. If one follows the approach of Israelachvili (1991) and Boal (2000), one takes only the leading $1/A_m$ dependent term of the repulsion, and assumes constant interfacial tension as shown in Fig. I.4.2. Yet, the nature of the lipid interface also causes the interfacial tension to be different for different lipid packing densities. At high packing densities the headgroup coverage is good, and most of the interactions are between water molecules and lipid headgroups. With an increasing area per molecule the umbrella effect of headgroup coverage gets weaker, and more and more water starts pouring into the acyl chain region. Additionally, one expects the geometry of the interface to change, from closely packed rods towards closely packed cones, the headgroup being the top of the cone. The latter effect would be expected to result due to the increasing level of acyl chain *trans→gauche* isomerization, the increasing cross-sectional area of the acyl chains near the bilayer centre, and the linkage of two acyl chains to the two contiguous carbons of the glycerol. These effects (Fig. I.4.3.) will lead to a change in the interfacial tension with a changing area. It is of interest to notice that osmotic type equations of state have been explored for lipid films (Feng, 1999).

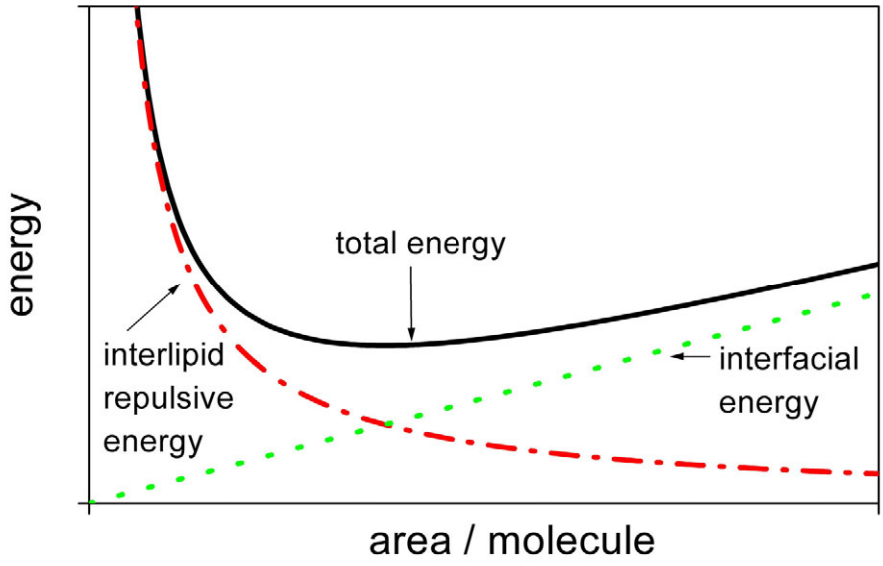

Figure I.4.2. The balance of the interfacial tension and interlipid repulsion in the bilayer, following Israelachvili (1991) and Boal (2000). From Kinnunen and Alakoskela, *manuscript in preparation.*

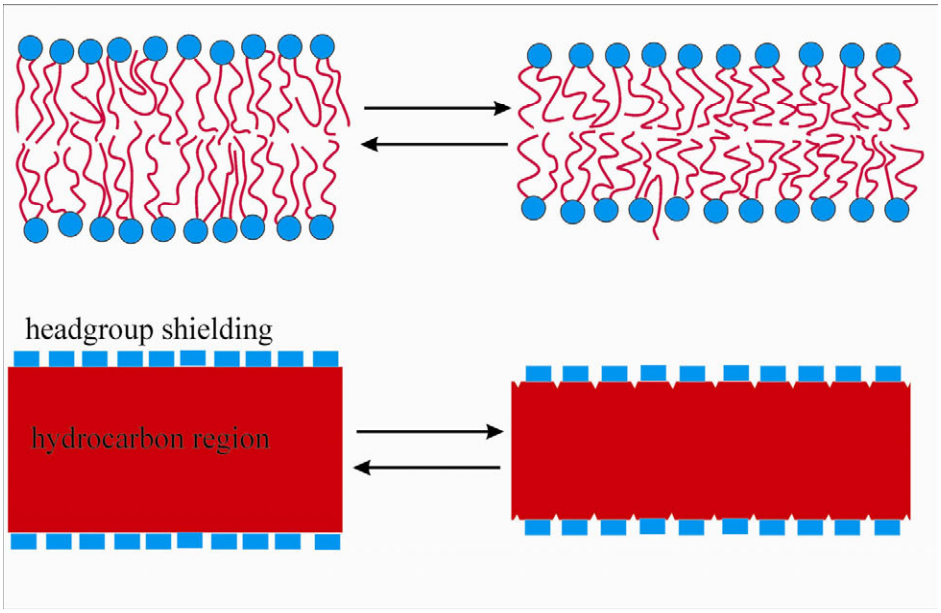

Figure I.4.3. As the area of the lipid bilayer increases, the fluid bilayer behaves as an almost incompressible fluid, and the increase in area is balanced by a decrease in thickness. The area covered by headgroups does not change to the same extent as the bilayer surface area. Accordingly, increasing the area exposes more hydrocarbon (red) phase to the surrounding water. On the other hand, the configurational entropy of the acyl chains as well as direct repulsion increases as the bilayer area decreases. Modified from Kinnunen and Alakoskela, *manuscript in preparation.*

4.2. Lateral pressure profile

As discussed in the previous chapter, the bilayers are in equilibrium and the internal pressure balances the interfacial tension. Yet, tension and pressure were considered only as truly two-dimensional properties. In other words, not only were they taken as the two-dimensional tension and pressure in a sense that they are of the form force / contour length, but also they were thought of as properties projected on the bilayer plane, their locations in the bilayer were not considered. However, even at first thought one realises that the interfacial tension acts in the interface, while much of the interlipid repulsion and consequent internal pressure acts in the hydrocarbon part of the interface. Thus, to describe this, one needs to consider the bilayer lateral pressure profile, i.e. the real components of the lateral (in the xy-plane) pressure and tension at different points of the z-axis, where the z-axis is along the bilayer normal.

The lateral pressure profile and its possible effects on proteins were introduced by Cantor (1997a, 1997b). Basically, when integrating the lateral components over the whole z-axis, one always get zero, but the crucial idea is that very different profiles can achieve this result, and these different kinds of profiles are coupled to different kinds of stresses in membranes [e.g. for slightly cone-shaped lipids (shape that predisposes to bilayer→H_{II} transition) much of the pressure is due to components close to bilayer centre, and for slightly inverted cone-shaped lipids (shape that predisposes to bilayer→worm-

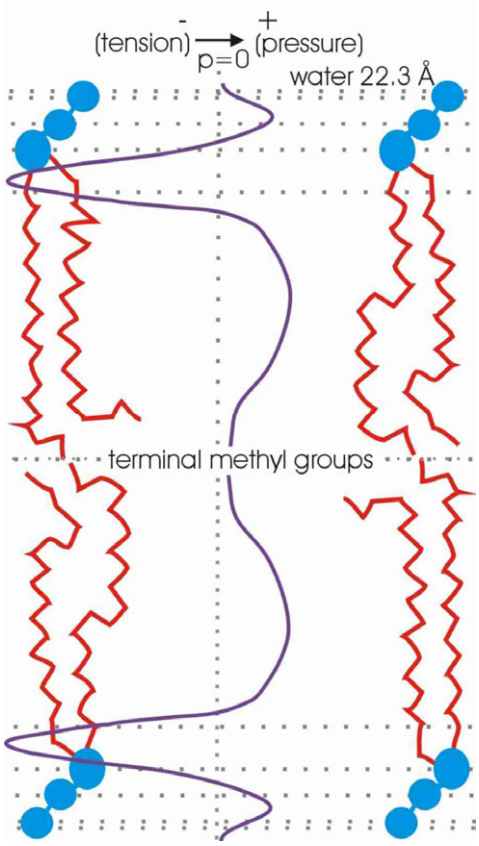

Figure I.4.4. A schematic presentation of the lateral pressure profile. The dotted vertical line in the middle shows the position of the zero pressure/tension. The violet continuous line presents the overall features of interfacial tension/pressure. A deviation to the right of the dotted line reveals repulsive interactions (pressure), and to the left attractive interactions (tension). The integrated areas to the left and right of the zero line equal each other if the bilayer is in equilibrium with respect to the area. When moving from the water phase into the hydrocarbon phase the first positive bump in the pressure/tension line shows the interheadgroup repulsion, which should mostly consist of electrostatic (e.g. charge–charge or dipole–dipole) and steric repulsion. The large negative region of the curve shows the interfacial tension, and the deeper positive part of the curve shows the repulsion between the acyl chains. A change in one of the components must be balanced by the others. Accordingly, the increase in headgroup repulsion is likely to both relieve the acyl chain pressure somewhat due to an increase in area, and to increase interfacial tension as the hydrocarbon part of the membrane will be shielded from water to a lesser extent. The small interheadgroup repulsion, large interfacial tension and strong pressure at the acyl chain region favour transitions from bilayers to nonlamellar phases with small a interfacial area, e.g. inverted hexagonal phase, and of course the opposite goes for the nonlamellar phases with a large headgroup area.

like micelles transition) much of the pressure would be due to components closer to the interface. In Fig. I.4.4. is a schematic presentation of a lateral pressure profile.

What makes the lateral pressure profile interesting is the fact that the lateral force components as well as possible changes in them are immense, they correspond to hundreds of atmospheres (Cantor, 1997a-b; 1999a-c). Thus, if different states, such as active and inactive states of an integral membrane protein are coupled to a conformational change that alters their cross-sectional area differently at different depths, one would expect the conformational balance of such a protein to be affected by a change in the lateral pressure profile (Cantor, 1997b; Cantor, 1999b). While this interesting and important idea has been a subject of theoretical study, and has been suggested to be the mechanism for anaesthetic action and endogenous desensitation of postsynaptic receptors (Cantor, 2003), there are unfortunately no experimental methods that could be used to directly measure lateral pressure profile with sufficient precision. Lately this restriction has somewhat been relieved by atomistic MD simulations allowing for the calculation of lateral pressure profile in a setting more realistic than earlier theoretical considerations (Gullingsrud and Schulten, 2004; Lindahl and Edholm, 2000). While the lateral pressure profile itself is not accessible to study, its integral moments, the first of which is related to the splay curvature elastic modulus and spontaneous curvature, and the second to the Gaussian curvature modulus (Cantor, 1999b), can both be calculated from simulations (Gullingsrud and Schulten, 2004) and are accessible to experiments as well. In reality, the integral moments may tell little about the profile. In one of the most interesting molecular dynamics simulation studies of lipid bilayers cholesterol was found to induce a very complex, multipeak profile (Patra, 2005). More specifically, in the presence of cholesterol there is an alternating array of very strong tension (contracting) and pressure (expanding) components. Importantly, the study by Patra suggests that proteins may have complex lateral pressure profiles as well, and in this case compound-specific changes in lateral pressure profile could lead to specific effects on proteins without binding of the compound to proteins. Additionally, there is a distinct possibility of kinetic effects, e.g. the simulated bumpy profile by cholesterol is bound to offer multiple kinetic traps to a protein whose conformational change includes changes in the positions of amino acids in the hydrocarbon region of the bilayer. While further studies are required to assess the importance of the lateral pressure profile in biomembranes, it is becoming accepted that at least for mechanosensitive channels the lateral pressure profile may be of primary importance (Kung, 2005).

5. Membrane dipole potential

As the lipid molecules form into a bilayer, they come together in a certain orientation: their acyl chains are directed towards the centre of the bilayer and their headgroups are on the surface. Water molecules have interactions with the polar groups of lipid molecules, and there is hydration water surrounding the bilayer surface, as discussed. As the lipids themselves are driven by hydrophobic interactions to form the bilayer, so are the dipoles of the lipids and those of interfacial water molecules forced into a preferred orientation with respect to the membrane normal, resulting in layers of positive and negative partial charges within the membrane (Brockman, 1994; Cafiso, 1998). The resulting potential difference between layers is called the membrane dipole potential. In

the case of fluid phase phosphatidylcholines, the potential difference is at least approximately 200–300 mV (Gawrisch, 1992; Franklin and Cafiso, 1993), mostly over a region of roughly 1.0 nm (Marrink et al., 1996), generating an electric field of at least approx. 2–3 $\times 10^8$ V/m, which is roughly 10 times the magnitude of the electric fields usually generated by a transmembrane potential difference. The complete electric potential of the lipid bilayers is usually in simplistic handling divided to surface potential, dipole potential and (trans)membrane potential (Fig. I.5.1). First is attributed to the surface net charge, second to the dipoles, and the last to the ion excess in bulk.

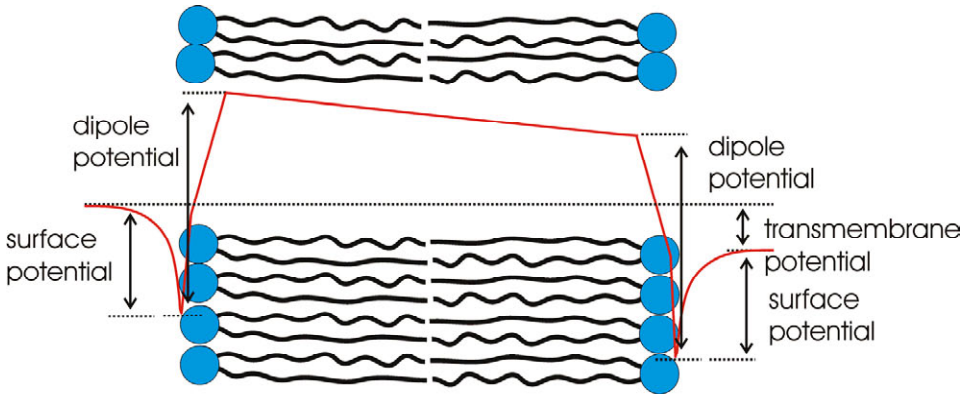

Figure I.5.1. The electric potential gradients in a bilayer. The transmembrane potential is the potential difference between the bulk liquids, the surface potential is the potential due to surface charges, and the dipole potential results from the partial charges of dipoles present in the bilayer. The potential gradient inside the hydrophobic part of the bilayer is sometimes coined diffusion potential.

5.1. Experimental approaches in the study of dipole potential

There are two major ways to do the independent measurement of the dipole potential for lipid films, one uses lipid monolayers at air/water interface, the other estimates the dipole potential essentially by fitting the dipole potential to a value that explains the different permeability of bilayers to anions and cations.

The measurement of dipole potential in monolayers most often uses a vibrating plate or Kelvin method, in which applied static voltage is used to suppress the potential difference arising due to dipoles at the interface (for more information, see e.g. Brockman, 1994; Clarke, 2001). With this approach one typically records dipole potentials in the range of 400–500 mV for phosphocholine monolayers at packing densities similar to those expected in bilayers (Brockman, 1994). The positive sign is used to indicate that the inside of the membrane is positive with respect to the bulk water or interface. The sign convention varies in works by different authors, particularly in the MD simulation field, where either the center of membrane is set at zero potential (resulting in negative dipole potential values for PCs) or the center of the water lamella is set at zero potential, resulting in the same sign convention as adopted here and in almost all experimental work.

The method based on different permeability or conductance uses structurally similar hydrophobic anions and cations, typically tetraphenylphosphonium (TPP^+), tetraphenylarsonium (TPA^+), and tetraphenylborate (TPB^-), and supposes that the differences in conductivities are solely due to the difference in dipole potential (see Schramberger and Clarke, 2002). Different simplifications have been used, either continuum approach (see e.g. Gawrisch et al., 1992) or the square point-dipole lattice approximation (Flewelling and Hubbell, 1986; Franklin and Cafiso, 1993). Typically these measurements give dipole potential of phosphocholine bilayers a value of 200–280 mV (Flewelling and Hubbell, 1986; Gawrisch et al., 1992; Franklin and Cafiso, 1993).

There is thus a discrepancy of 120–280 mV between the values of phosphatidylcholine dipole potential produced by monolayer and conductance approaches. Two possible, mutually nonexclusive possibilities to explain this difference have been suggested. First, if one extrapolates the monolayer surface dipole potential vs. the lipid surface density curves to zero surface density, one obtains a nonzero value whose sign and magnitude depends on the lipid composition, having values -75–144 (Smaby and Brockman, 1990; Brockman, 1994). For PCs this value, Ψ_0, is in the range of 93–126 mV. This potential difference has been attributed to the different water orientation between air/water and lipid/water interfaces, essentially reflecting lipid- and particularly headgroup-specific difference between water organization at the pure interface and the lipid covered interface (Smaby and Brockman, 1990; Brockman, 1994). This assumption is supported by proton conductance experiments, as a drastic increase in monolayer lateral proton conductance coincides with the appearance of Ψ_0 (see Brockman, 1994; Oliveira and Bonardi, 1997, and references therein). Another interesting feature is that the clean water surface itself has an electric potential. The availability of hydrogen bonding and other interactions with other water molecules only on the water side of the air/water interface restricts the orientation of interfacial water molecules, and experiments and modelling appear to suggest that the dipole moment of water is almost parallel to the plane of the interface, the negative oxygen end directed slightly (8°) towards air (Goh et al., 1988; Sokhan and Tildeley, 1997). This orientation would result in a negative dipole potential for the clean air/water interface. In the measurement of monolayer dipole potential the sensor is zeroed against the free air/water interface, and if the initial negative dipole potential disappears upon the introduction of lipid into the interface, one would expect additional positive contribution to the dipole potential of monolayer so measured compared to the dipole potential of bilayers. Unfortunately, due to difficulties deriving largely from the lack of reference points, the attempts to measure the dipole potential of clean air/water interface have been unsuccessful, producing wildly different values, -500 to 1100 mV (Parfenyuk, 2002). Nevertheless, most values are between -200 and -100 mV (Paluch, 2000), and in his review of literature Parfenyuk (2002) came to conclude that most reliable estimate is approx. -100 mV, negative towards gas phase and positive towards water. Incidentally, this corresponds fairly well with the estimate for PCs and PEs by Brockman (1994). Accordingly, in measurements of monolayer surface potential the zero value is set at an electric surface potential having -100 mV difference to bulk potential, and e.g. the measured surface dipole potential of 400 mV should correspond to approx. 300 mV when the bulk water is chosen as reference point. Interestingly, the role of hydration and the water structure around the zwitterionic headgroups deserves a closer look, as charged phospholipids such as POPS and POPA have Ψ_0 of nearly zero or even slightly negative.

Lipid charges may disrupt the arrangement of the water network that likely exists for PC, and may explain lower Ψ_0 values.

If the suggestion of the role of Ψ_0 is correct, then one would expect the orientations of water molecules in negatively charged interfaces to produce nearly the same dipole contribution as on the pure air/water surface, whereas zwitterionic lipids would basically abolish the existing orientation. As odd as the similar orientational distribution at a pure surface and at the surface of anionic phospholipids sounds at first, there are some factors to reduce this oddness. Both experimental evidence as well as theory to explain Jones-Ray effect suggest that in pure water hydroxyl ions are enriched in the surface, although the surface charge density is lower than expected for anionic lipids. The surface potential of pure water might also be partly derived from these anions (Karraker and Radke, 2002). Thus, the replacement of an anion layer by an anion layer of higher charge density might indeed be expected to have a smaller effect than replacement of an anion layer by a zwitterion layer. Although I am not aware of any studies addressing the topic, it clearly would be of interest to test the difference in dipole potential in bilayers and monolayers of different compositions. Obviously, the use of charged lipids would introduce complications to the ion conductance approach, and would also raise questions about the reliability of the fluorescence studies addressing this discrepancy.

The other suggestion for the origin of discrepancy questions the basic assumptions of the ion conductance method. The ion conductance method presupposes that for the ions used, due to the structural similarity and shielding of the large phenyl groups, interactions related to ion size and solvation are equal irrespective of the charge of the ion. Nevertheless, it has been pointed out that the electronegativity difference between carbon and the atom at the center of ions tetraphenylphosphonium (TPP^+), tetraphenylarsonium (TPA^+), and tetraphenylborate (TPB^-) cause the positive charge to concentrate to the center, and the negative charge of TPB^- to be distributed to the phenyl rings. This causes the solvation energy to be considerably different for the ions in question, and when this is taken into account in the dipole potential calculation based on conductance, the dipole potential values for phosphocholine bilayers are approx. 120 mV larger than those without the correction, giving values of approx. 340 mV (Schramberger and Clarke, 2002). Notably, the model used in the ion conduction method — a continuum or a point dipole lattice — affects the magnitude obtained. The values of the point dipole lattice model (Franklin and Cafiso, 1993), 280 mV for egg-PC, being approx. 50 mV larger than those of continuum approach (Gawrisch et al., 1992), which suggests that the continuum approach itself also underestimates the dipole potential. This could indicate that a somewhat higher dipole potential than 340 mV should be assigned. Additionally, one should note that there is a considerable uncertainty in the exact value of the solvation energy difference. Thus uncertainty in the exact value of the dipole potential correction due to this second effect is very large, too. Yet, we have two plausible contributions to account for the dipole potential difference between monolayers and bilayers.

The two methods discussed above, especially the ion conduction method giving values for bilayers, provide calibration points for assays that are unable to even indirectly produce the value of the dipole potential without such calibration points. Easy-to-use and highly accurate fluorescence spectroscopic methods are the most important of these methods, and the most important individual probe is di-8-ANEPPS, that is relatively insensitive to contributions other than dipole potential (Gross et al., 1994).

5.2. On the submolecular level origins of dipole potential

The contribution of interfacial water and the different parts of the phospholipid molecules to dipole potential has been studied extensively. The main contributors are headgroups, carbonyl groups, terminal methyl groups, and interfacial water molecules (Brockman, 1994; Langner and Kubica. 1999). While these contributions are directly accessible in molecular dynamics simulations, the experimental attempts have often used simple models. Molecular dynamics simulations (Chiu et al., 1995; Marrink et al., 1996; Stern and Feller, 2003) suggest that in DMPC and DPPC bilayers Ψ originates from the oriented water molecules overbalancing the contribution of the lipid dipole moments. In the simulations the absolute individual contributions of lipid and water were many times greater than that of the net dipole potential, and the overbalancing by water is caused by the steric constraints imposed by the optimization of hydrogen bonding between the water molecules intercalated into the interfacial region. The role of the water molecules is also (though weakly) supported by the close resemblance of the curves of Ψ vs. charge density for lipid monolayers and for the metal/water interface, the latter of which is known to be produced by the polarization of water molecules (Moncelli et al., 1998). Now remains the question how the dipole moments of different parts of lipids contribute to the overbalancing effect. In some cases elegant experiments in bilayers have proved possible. Remarkably, 1,2-dihexadecyl-*sn*-glycero-3-phosphocholine (DHPC) with ether-bonded alkyl chains instead of the ester-bonded alkyl chains of DPPC Ψ is approximately 118±15 mV smaller in absolute magnitude, 109 mV instead of 227 mV (the values are based on continuum approach ion conduction, Gawrisch et al., 1992). Obviously, with the disappearance of carbonyls there is either a direct contribution, or a disappearance of orientation in water molecules polarized by the carbonyls. Interestingly, the solvation pressure changes in most cases closely parallel the dipole potential changes (Simon and McIntosh, 1989; McIntosh et al., 1989; Simon et al., 1992), but the solvation pressure is nearly equal for DPPC and DHPC despite the large difference in dipole potential, suggesting that the outer water layers for DPPC and DHPC are similar (Gawrish et al., 1992). For most purposes lipid derivatives of such similarity are not available, and the simplified models based on Helmholtz equation have been used in monolayer studies. One should notice that all of these studies are based on an assumption that dipole potential simply consists of the sum of the contribution of the dipoles of molecules or their parts. The simplest form of model is

$$\Psi = \frac{\mu_{\perp}}{A\varepsilon},$$

in which $\mu_{\perp}$ is the dipole moment perpendicular to the surface, A is the average area per molecule and ε is the permittivity (see Brockman, 1994, and Taylor, 2000). Somewhat more complicated models divide the dipole potential to two or three independent contributions. In the latter case one is from the water dipoles, one from the interfacial region dipoles and one from hydrocarbon region dipoles, all with different effective relative permittivities (ε_1, ε_2, ε_3) assigned to these regions (see Brockman, 1994, and particularly Taylor, 2000):

$$\Psi = \frac{1}{A\varepsilon_0}\left(\frac{\mu_{\perp,1}}{\varepsilon_1} + \frac{\mu_{\perp,2}}{\varepsilon_2} + \frac{\mu_{\perp,3}}{\varepsilon_3}\right).$$

Often it is assumed that the region closest to the lipid/water interface makes little contribution to Ψ as the relative permittivity is highest over there. With respect to the charged lipids one would also expect a significant contribution from the diffuse double layer. In an attempt to evaluate the contribution from terminal methyl groups Vogel and Möbius (1988) came to the conclusion that terminal methyl groups are the most important source of dipole potential by comparing stearic acid monolayers to a 1:1 mixture of corresponding alkane and dicarboxylic acid, assuming that the organization is the same, as the compression isotherms for the two compositions were very similar. Further, they assumed that the opposing methyl groups of the alkane will result to zero. However, even within this simplified scheme their assumption is not truly valid, as the effective permittivity at different ends of alkane may be different, and the conformation of the carboxylic acid headgroup may differ, as has been discussed (see Brockman, 1994; Taylor, 2000). Additionally, the effective relative permittivities are dependent on the area (Taylor and Bayes, 1994; 1999; Taylor, 2000), which complicates the data handling. This may partly contribute to the deviation of apparent dipole moment as estimated based on the above formulas, although allowing for Ψ_0 rather good results with a constant dipole moment are obtained (Smaby and Brockman, 1990; Brockman, 1994). Obviously, an additional contribution would be expected by area-dependent orientation changes of the dipoles. One valid estimate appears to be the independence of long (>10 carbons) alkyl chain terminal dipoles and the lipid/water interface, both theoretically (Taylor and Bayes, 1994; 1999; Taylor, 2000) and experimentally (Petrov and Möbius, 1996). Considering the complications it is not surprising that results in different experimental systems are different. By studying black lipid membranes with chain carbons at various positions substituted for sulfurs Peterson et al. (2002) suggested that terminal methyl groups play no role in dipole potential, arriving at an opposite conclusion compared to that of Vogel and Möbius. Their rationale was that in fluid bilayers the order decreases more and more towards the center, randomizing the orientation. Additionally one may note that in atomistic MD simulations a central bump has been observed in the lateral pressure profile. This bump is suggested to derive from the overlap of terminal methyl groups, and thus, there is also a real possibility that in bilayers there is an additional neutralization of the terminal methyl group dipole moments by the terminal methyl group dipole moments of the other leaflet. However, it is obvious that in some cases groups deep within bilayer can have a significant effect on dipole potential. In fact, phospholipids with fluorinated alkyl chains show not only a decrease in dipole potential, but reversal of its sign. Results by McIntosh et al. (1996) can be summarized as follows: Ψ=415 mV for ordinary (egg-)PC, Ψ=-180 mV for one fluorinated acyl chain, and Ψ=-680 mV for PC with two fluorinated acyl chains. Similar results have been produced by MD simulations of fluorinated PCs by Smondyrev and Berkowitz (1999) who use the convention of setting the potential at zero in the center of the bilayer, and who also rather confusingly use a different origin for z-axis depending on the parameter displayed (bilayer center for most, water layer center for dipole potential). The simulation data suggest that the contribution of the $-CH_3$ group is very small, <100 mV, whereas the contribution of $-CF_3$ is much larger, approx. 1000 mV, and considerable compared to the total dipole potentials of 5000–6000 mV of water and lipids both (signs are opposite for dipole potentials due to lipid and water; the lipid contribution originates mostly from the phosphocholine dipoles). The much larger contribution of the fluorinated group was able to tip the total dipole potential from 650 mV to -300 mV in the simulation, with small effects on surface

water or headgroups. Nevertheless, the simulation predicts much more fine structure for fluorinated bilayers that show interfacial potentials of approx. 700 mV compared to bulk water, as in nonfluorinated case, but then decreasing to the value of -300 mV only close to the bilayer center. This complex profile has been suggested to partly explain the good permeability barrier formed by fluorinated lipids, as they contain potential energy barriers for both negatively and positively charged moieties. Other experimental work based on above mentioned simple models of monolayer surface potential have suggested a roughly equal contribution of headgroups and acyl chains to the total dipole potential (see references in Taylor and Bayes, 1994 and Taylor, 2000). The simulations are not without problems, as they do not account for electronic polarizability, which may contribute to the results, particularly in the acyl chain region (Taylor, 2000; Taylor and Bayes 1994; 1999). Nevertheless, such effects are likely not too extensive, and the considerable role suggested for terminal methyls based on monolayers could derive to a large extent from forcing the results into a simple model not accounting for the full complexity of dipole potential. Of course, some genuine differences in monolayers and bilayers are possible, as intercalation of methyl ends does not occur in monolayers at gas/water interface as it does in bilayers. Yet, we may conclude that for bilayers of normal, unmodified phospholipids, the distal parts of alkyl chains make a relatively small direct contribution to Ψ. They do make indirect contributions, however, as they affect the equilibrium molecular area, which is connected to the value of dipole potential (Clarke, 1997). Additionally, carbon—carbon double and triple bonds close to the lipid/water interface have great effects. In ceramides dipole potential nearly doubles (277→546 mV) if the *trans* double bond is introduced to the immediate vicinity of the interface. The effect is even larger with a triple bond and the effect of the double bond decreases if it is farther away from interface. These results are likely related to the large polarizability of these bonds and their interaction with water, as they cannot be explained by packing changes observed (Brockman et al., 2004).

As the alkyl chains have impact on Ψ only in special circumstances, the interfacial region is obviously the most important determinant of dipole potential. The role of the carbonyls has been emphasized (Franklin and Cafiso, 1993; Gawrish et al. 1992), and the replacement of an ester bond lipid by an ether bond lipid decreases the dipole potential by half (Gawrisch et al., 1992). This effect is related to the water molecules polarized by carbonyls, and by adding trehalose the polarized water molecules can be released, resulting in a considerable decrease in dipole potential, roughly 70 mV at 0.1 M trehalose (Luzardo et al., 2000), although there is a complex area dependence of the effect (Lambruschini et al., 2000). In simulations trehalose was found to induce a 27 mV decrease at approx. the same concentration, originating from a large decrease in the water component from 4.9 V to 3.9 V, a large change of the lipid component from -4.4 V to -3.8 V, and finally a 0.4 V new component due to the trehalose hydroxyl group orientations, that are hydrogen-bonded to the membrane through a mediating water layer (Villareal et al., 2004). Phosphate groups and water polarized to them also make a considerable contribution to dipole potential. Indeed, a dipole potential decreasing substance phloretin appears to associate with the phosphate groups at lower mole fractions, having little effect on surface pressure of monolayers (Cseh and Benz, 1998). The effect of this compound on dipole potential appears to be derived mostly from its interactions with the phosphates (Bernik et al., 2001; Diaz et al., 2001), hydrogen-bonding with them, and changing their hydration, along with changes in the P-N dipole orientation (Jendrasiak, 1997; Bernik et al., 2001; Diaz et al., 2001). Incidentally,

phloretin provides one example of complications found in binding to membranes, not following the simple model of adding dipoles into monolayers (Cseh and Benz, 1998), and phloretin has ability to adopt different conformations (having different dipole moments) in membranes (Cseh et al., 2000). However, interactions with phosphate are the key. This is illustrated by the effects of phloretin on the dipole potentials of different monolayers. Mixing phloretin with phospholipid to give a 1:1 mixture decreases the dipole potential of DMPC and DMPG monolayers by approx. 150 mV and 60 mV, and has no effect on dipole potentials of DMPE monolayers. These effects have been suggested to derive from the participation of the phosphate moiety to stronger interheadgroup interactions in DMPE and DMPG monolayers, not allowing for phloretin to bond to phosphate (Lairion and Disalvo, 2004). Even within the simple model there are complications, as the effective dipole moments of the compound residing in the bilayer are modulated by the local dielectric constant or permittivity, and the smaller the dielectric constant, the larger the effective dipole moment is relative to the real dipole moment. Additionally, as the dipoles are polarizable, they themselves contribute to the permittivity. This necessitates the use of models that allow the local dielectric constant to change as a function of the number of bound dipoles (Qin et al., 1995). Likewise, the orientation of the real dipole of the compound determines the length of the projection in the direction of the membrane normal, i.e. the normal component of the real dipole moment. Orientation may also change as function of the number of bound dipoles. A further complication is provided by the anisotropic nature of the membrane. (And let us restrict ourselves to the static permittivities.) The relative permittivity or the dielectric constant ε of the membrane is not only a function of the position along the membrane normal or z-axis, i.e. $\varepsilon(z)$, but is a tensor quantity. For a given point in the membrane the relative permittivity of the surroundings should be different in different directions (e.g. in the membrane or xy-plane, towards negative z-axis, towards positive z-axis etc.). The rationale for this is easy to understand – let us consider the strong phosphocholine dipoles of lipid headgroups, which for liquid-crystalline bilayers are free to rotate in the plane of the bilayer. For a charge or dipole in that plane the reorientation of phosphocholine dipoles can very efficiently neutralize the xy-components, but poorly the z-components, leaving them to interact with water and carbonyls. Support for the anisotropic nature of the local permittivity is provided by more compelling facts than these simplified gedanken experiments. Theoretical work based on continuum assumptions (Raudino and Mauzerall, 1984), anisotropic fluctuations of interfacial molecules in MD simulations (Shinoda et al., 1998), and the calculation of dielectric constants from the MD simulations (Stern and Feller, 2003) all support anisotropic dielectric permittivity. The calculations of Stern and Feller (2003) suggested that in the phosphocholine layer the dielectric constant in the bilayer or xy-plane is approx. 350, roughly four times that of water, and while the simulation did not allow for an exact calculation of the normal component, it could be as low as 8 in the same layer. Qualitatively similar results were obtained by Wohlert and Edholm (2004), who found the lateral permittivity equal to that of bulk water, and the vertical permittivity from -11 to -5, indicating the overbalancing or overpolarization. This together with a strong z-dependency, and the possible need to consider dynamic dielectric constants could easily explain the large spread of values produced for the interfacial dielectric constant by different experimental approaches.

5.3. Coupling between dipole potential, hydration, and headgroup orientation

As we already discussed, several studies have demonstrated a correlation between the dipole potentials measured from monolayers and the hydration pressure between bilayers (Simon and McIntosh, 1989; Simon et al., 1992; Jendrasiak et al., 1997). This is hardly surprising considering the origin of the dipole potential, which is deeply connected to the dipole moments of the interfacial hydration water. Nevertheless, modifications of the dipoles deeper in the bilayer lead to a decoupling of these two properties, as exemplified by the ether lipid analogue of DPPC, 1,2-dihexadecanyl-*sn*-glycero-3-phosphocholine (DHPC). If DPPC is replaced by DHPC the hydration pressure remains nearly unaltered despite the 118 mV decrease in dipole potential (Gawrisch et al., 1992). Similarly, the hydration pressure for PCs with fluorinated acyl chains is nearly equal to the hydration pressure of ordinary PCs (McIntosh et al., 1996).

Phosphocholine headgroup can easily change its orientation, and it is a very strong dipole. Not surprisingly then, the phosphocholine headgroup orientation is known to be modulated for example by the surface potential due to external electric fields (Stulen, 1981), the charged species in the membrane (Scherer and Seelig, 1989), and due to the dipole potential altering substances like phloretin (Bechinger and Seelig, 1991a) and DHPC (Siminovitch et al., 1983).

Partial charges in the interface can be screened to some extent by both cations and anions. While cations screen the negative partial charges on the outer surface, decreasing dipole potential, anions tend to screen the inner layer of positive partial charges, also decreasing dipole potential (Clarke and Lüpfert, 1999). Importantly, the screening is not nearly complete, and there still remains an excess of partial charges, as evidenced by the fact that the dipole potential decreases only slightly upon the increase of the cation concentration in the bulk phase. This shows how the dipole potential is coupled to the ζ-potential, and indeed the increasing Ψ appears to promote anion binding. This keeps with the coincidence of maxima in Ψ and minima in ζ-potential in binary phospholipid membranes (Luzardo et al., 1998).

5.4. Interplay of proteins, drugs, ions, and dipole potential

Although the role of dipole potential in biological systems is still unclear, data about the interactions between the dipole potential and molecules associated to cell membranes is slowly accumulating. The binding of a molecule to a bilayer, or a conformational change of a protein likely changes the direction or magnitude of the dipole moment component in the direction of bilayer normal. Thus a binding reaction or a conformational change of a protein would change Ψ. From this it directly follows that these reactions in turn should be affected by changes in Ψ. Accordingly, the question is not about whether the various reactions in the membrane interface are affected by Ψ, but rather is the magnitude of the effect significant. This, of course, depends not only on the magnitude of $\Delta\Psi$, but also on the magnitude of the change in normal component of the dipole moment that takes place during the reaction, and the magnitude of the total free energy change of the interfacial reaction, such as conformational change of an integral membrane protein.

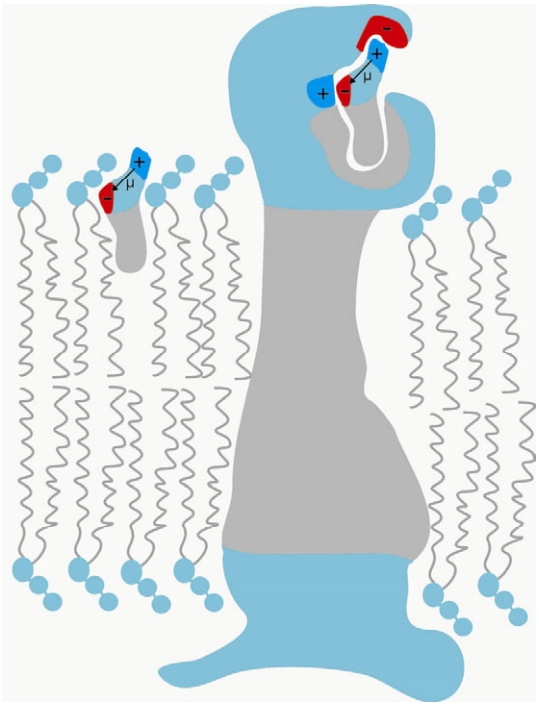

Figure I.5.2. A possibility behind correlation between dipole potential and biological effect in a situation in which effect is actually mediated by direct binding. The hydrophilic parts are shown in light blue, and the hydrophobic parts in grey. Areas of positive and negative partial charge are shown in blue and red, respectively, but only for the drug and the protein binding site. See text for details.

Studies on the effects of other membrane physical properties on integral membrane proteins are largely compromised by the fact that the purified integral membrane proteins are either not available in large amounts or are not easily reconstituted in vesicles of known lipid composition. The studies on the effects of Ψ make no exception, and the work that has been done using utterly complex, practically unknown systems like cells is always problematic, as it is at best difficult and usually impossible to say whether the observed effect is due to indirect membrane effects such as altering Ψ, or due to direct or indirect effects due to binding to unknown (probably low-affinity) binding sites in the protein of study or proteins interacting with it. Of course, it should be kept in mind that the confounding effect works both ways, and unless the binding site is actually known, the effect of a substance on a protein might be indirect. Further is should be noted that the dose-response curves are not proof of binding. For some more complex events like the effects of Ψ on whole organelles, cells, or organisms only circumstantial evidence exists, mostly consisting of studies showing a correlation for the effect under study and the effect of inducing molecules on dipole potential. These studies are especially problematic, as 1) correlation is not proof of causality, and 2) it would be within reason that molecules binding to a binding site are of intermediate polarity and have their dipole moment in certain orientation with respect to the polarity distribution along the molecule, so as to maximize the hydrophobic and dipolar interactions with the binding site. In this case, it would also be reasonable that these compounds would have similar effects on the dipole potential as well (see Fig. I.5.2.). One possible, yet laborious way to circumvent this problem would be the use of enantiomers, as the dipole potential effect is likely to be almost same, whereas binding is expected to have large differences. In effect, this approach would be similar to the approach suggested by Douglas Covey, which is to use enantiomer of cholesterol to discriminate between the effects on the membrane physical

properties and the effects on the binding to proteins (Westover and Covey, 2004). However, this would require one to synthesize the enantiomers of common dipole potential modulators such as 6-ketocholestanol and phloretin-related compounds (phloretin itself does not have a chiral carbon). Accordingly, while taking substances with similar effects and checking their effect on Ψ can never provide any evidence for the membrane-mediated action, the lack of correlation can at least disprove effect mediated by the membrane dipole potential. Somewhat more plausible is the link between causality and correlation in the cases in which one selects structurally dissimilar strong potentiators and attenuators of dipole potential, and finds that they produce opposite effects, as these substances were selected only due to their effect on dipole potential, and it would be a great and unfortunate coincidence if they would bind to a binding site, one acting as agonist and the other as antagonist. Yet, it is impossible to completely rule out this, especially in unknown, complex systems like cells, though such studies would enhance their weight as evidence if they would use several different dipole potential modulators. Keeping these difficulties in mind, we may review the interactions between the membrane dipole potential and various components at different levels of organization and study.

One of the strongest dipole potential attenuators is phloretin, a compound naturally present in apples (Lee et al., 2003). The original interest in phloretin was due to the fact that it is a reversible inhibitor of hexose transport system of human erythrocytes. Interestingly, it was found to inhibit transbilayer movement of a number of compounds, such as glycerol, urea, water, and chloride, and phloretin has been shown to inhibit for example type 1 and 2 glucose transporters (GLUT1 and GLUT2) (see e.g. references in Fan et al., 2001), aquaporin-2 (Moshelion et al., 2002), UT-A urea channel (see e.g. Bagnasco et al., 2001), *H. pylori* urea permease VacA (Tombola et al., 2001), H^+:pantothenate transporter of *P. falciparum* (Saliba and Kirk, 2001), volume- and cAMP-sensitive chloride channels (Fan et al., 2001), mitochondrial F_0F_1-ATPase/ATP synthase (Zheng and Ramirez, 2000), protein kinase C (Ferriola et al., 1989), multi-drug-resistance mediator P-glycoprotein (Piwnica-Worms, 1995; Nguyen et al., 2003; Zhang and Morries, 2003), Na^+-PO_4^{3-}cotransporter (Peerce and Clarke, 2002; Peerce et al., 2003; Elmariah and Gunn, 2003), Na^+-glucose cotransporter SGLT-1 (Panayatova-Heiermann et al., 1999), GABA transporters of insects as well as humans (Rasola et al., 1995; Gao et al., 1999), and act as an antagonist of prostaglandin $F_{2\alpha}$ receptor (Kitanaka et al., 1993). Additionally, phloretin is an activator of large-conductance Ca^{2+}-activated K^+ channels (BK_{Ca}) (Klusemann and Meves, 1992; Koh et al., 1994). The drastically increased activation of Ca^{2+}-activated K^+ is largely derived from the decrease in the potential required for activation, and is tentatively attributed to a change in dipole potential (Koh et al., 1994). Phloretin is also an inhibitor of delayed rectifier K^+ channels I and F (Klusemann and Meves, 1992; Koh et al., 1994), and it inhibits the $K^+(Na^+)/H^+$ monovalent cation exchanger as well (Bernhardt et al., 1999). Interestingly, a delayed rectifier type of K^+ channel and A-type K^+ channel were recently shown to represent different functional states of the potassium channel due to a different lipid environment (Oliver et al., 2004). The list of phloretin effects on various transport mechanisms and enzymes is by no means complete, rather it is a result of a quick search in databases. While some effects, like those on hexose transport and protein kinase C, are likely to be mediated by direct binding, the large amount of different effects on very different proteins leads one to wonder whether some of these effects might be mediated by modulation of the membrane dipole potential by phloretin, instead of phloretin binding

specifically to tens of different proteins having only distantly related actions. Phloretin is also an uncoupler and an inhibitor of mitochondrial oxidative phosphorylation (De Jonge et al., 1983) and the widely-used carbonylcyanide phenylhydrazone uncouplers (known commonly by their abbreviations FCCP and CCCP) both decrease dipole potential (Reyes and Benos, 1984). Curiously, the strong dipole potential potentiator 6-ketocholestanol counteracts the effects of these uncouplers in both mammalian and plant mitochondria (Starkov et al., 1994; Vianello et al., 1995; Chávez et al., 1996; Cuéllar et al., 1997). Although the exact mechanism of recoupling remains open, all the authors have adopted the view that it must be by some modification of the lipid environment. It is tempting to speculate that somehow these effects might be connected, though even this correlation does not constitute conclusive evidence.

Now, in this brief review of the potential significance of the membrane dipole potential on the functioning of cells and cell components, we first take a look at simple

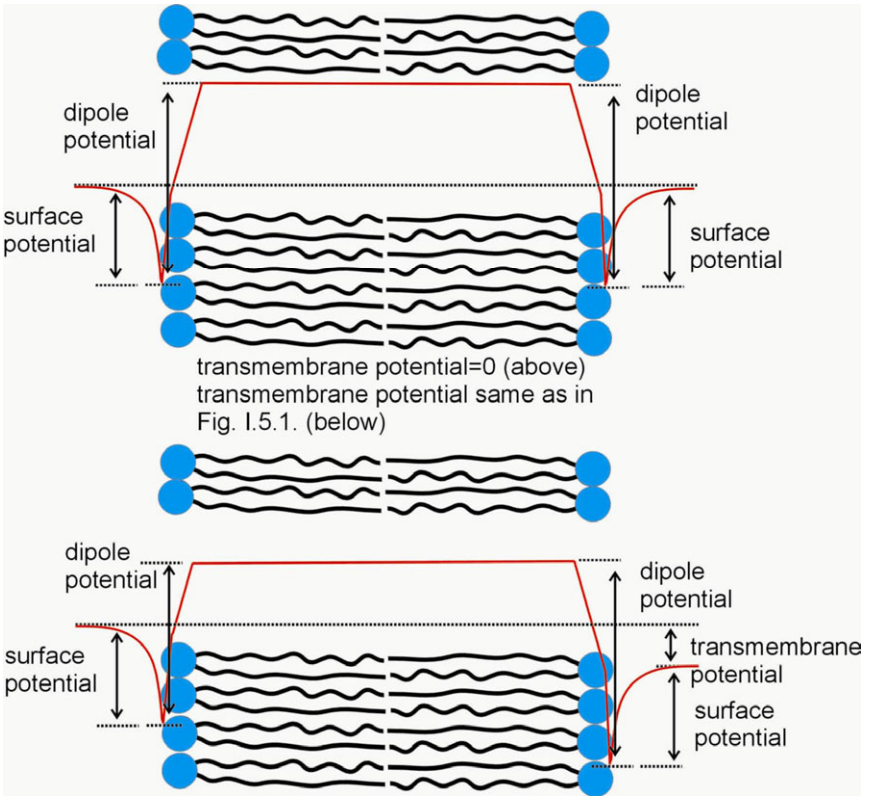

Figure I.5.3. The effect of the asymmetric dipole potential modulation on the intramembrane potential profile. See Fig. I.5.1. for the initial situation. The outside is on left, the inside on right. The upper panel shows the effect of the disappearance of transmembrane potential on the intramembrane potential profile in a symmetric bilayer. The lower panel shows the effect of a dipole potential decreasing substance added only on the outside. The effect on intramembrane potential profile is similar, and the effect should disappear when the flip-flop equalizes the membrane concentrations of the dipole potential modulators in inner and outer leaflet. Notice that the same effect on ithe ntramembrane potential profile should be produced by adding a dipole potential increasing substance on the inside.

model systems in which phloretin and 6-ketocholestanol are typically used as modulators of membrane dipole potential. The interest in the bilayer dipole potential was initially due to its effect on the transbilayer movement of hydrophobic cations and anions, and this difference has been used as one of the leading methods to measure the dipole potential. Accordingly, it is hardly surprising that the movement of biological ionophores with their associated ions is similarly affected by dipole potential (Bala et al., 2001). Similarly, it is no surprise that the decrease in (negative) dipole potential on the surface of membranes decreases membrane-binding of hydrophilic cations (Clarke and Lüpfert, 1999), but it could nevertheless be important, and this raises the question whether this might be part of the mechanism for the general inhibitory effect of phloretin on Na^+-coupled transport processes. While conduction of alkali metal ions is *not* expected to be greatly enhanced on the surface of the lipid bilayer like the conduction of H^+, it might be possible that to attain high transport rates the transporters need to use the bilayer reservoir of ions similar to the light-operated proton pump bacteriorhodopsin (Heberle et al., 1994). Alternatively, if the Na^+ binding site of transporters is deep enough in the interfacial region, the rates might be affected simply by a decrease in the local concentration of Na^+. However, these are purely speculative comments. Another binding effect is seen for interfacial peptides. The modulation of the dipole potential in simple model systems by phloretin and 6-ketocholestanol has been suggested to affect the binding of peptides to lipid bilayers and the folding of the peptide once bound to the bilayer (Cladera and O'Shea, 1998; Cladera et al., 1999). To this end, it is of interest to notice that two dipole potential decreasing compounds (phloretin and exifone) inhibit the interaction between the ABβ peptide and negatively charged lipid membranes, and also inhibit cytotoxicity of the ABβ peptide (Hertel et al., 1997), but it is not yet consistently proved that these effects are due to the dipole potential.

Effects of phloretin and 6-ketocholestanol on simple channels in model systems have been studied to some extent. Interesting discoveries include the findings that the dissociation kinetics of the gramicidin channel is oppositely affected by these dipole potentials modulators (Rokitskaya et al., 1997), and that phloretin decreases proton conductance and increases alkali ion conductance of the gramicidin channel, whereas the effects of 6-ketocholestanol are opposite (Rokitskaya et al., 2002). Another type of interaction seen in model systems and cells is the effect of dipole potential modulators on voltage-gated channels. Schagina et al. (2003) made the interesting discovery that the gating charge of a voltage-gated syringomyelin E channel is strongly affected by dipole potential modulators: dipole potential potentiators 6-ketocholestanol and RH-421 decrease the effective gating charge, even to the extent of changing the gating charge from positive to negative, while the dipole potential decreasing phloretin has an opposite effect. Another type of effect of the dipole potential on voltage-activated channels has been suggested to have its origin in the asymmetric of dipole potential in the bilayer after the addition of a dipole potential modulator on one side of the bilayer. The hallmark study addressed the effect of dipole-potential decreasing insecticide lindane on the voltage-activated Ca^{2+} channels (Silvestroni et al., 1997). After addition of lindane on the outside of sperm membranes, a transient, ion-independent depolarization is seen. This depolarization also triggers the voltage-activated Ca^{2+} channels, the result is an inflow of Ca^{2+} if Ca^{2+} is present in the outside buffer. The suggested mechanism involved is depicted in Fig. I.5.3. In the upper panel a change compared to Fig. I.5.1. is caused by membrane depolarization (left is outside, right is inside), resulting in a level intramembrane potential profile. In the lower panel, the same result i.e. level

intramembrane potential profile, is obtained by adding a dipole-potential-decreasing substance on the outside only. The latter effect is transient, as with time the fraction of dipole potential modulator becomes (nearly) equal in both leaflets, and only the total dipole potential is significantly changed in the end. This effect has also been suggested to account for the transient recoupling of oxidative phosphorylation by 6-ketocholestanol (Starkov et al., 1997) mentioned earlier. The transient intramembrane potential changes as modulators of protein function have received little attention though it would be especially attractive for biophysical studies, as one would expect a similar effect by the addition of a dipole-potential-increasing substance inside the cell, and an opposite effect by adding the dipole-potential-decreasing substance inside or the dipole-potential-increasing substance outside. Although this is merely speculation, it would be an appealing objective for future studies to apply this method to the voltage-gated channels that have been shown to be affected by dipole potential modulators. Another interesting effect is the finding that hybrid polar compounds that induce terminal cell line differentiation in tumours *increase* dipole potential (Herrero et al., 2000). However, it is highly uncertain that the effects on differentiation have any causal connection to dipole potential, especially since the dipole potential *decreasing* phloretin can also induce differentiation in certain cell lines (Takahari et al., 1998).

The role of dipole potential in the lives of cells remains uncertain and all the evidence is admittedly more or less circumstantial. Assuming the dipole potential had some physiological role in cell membranes, there should also be a way for a cell to regulate this potential. Of course, changes in lipid composition change the value of dipole potential. Changing the headgroups of lipids, changing the mole fraction of dipole-potential-increasing cholesterol, and changing the ratio of diacyl and plasmalogen phospholipids are all expected to affect dipole potential. A considerable increase in dipole potential is seen in response to the bradykinin receptor mediated activation of phospholipase C, as the dipole potential of the reaction product diacylglycerol is higher than that of the substrate phosphatidylinositol (Xu and Loew, 2003). Similar dipole potential changes have been suggested before, and the substrate species will determine both the sign and magnitude of change (Smaby and Brockman, 1990). Based on the effective dipole moments obtained from monolayer studies the major lipid species of plasma membranes, such as PCs and PEs, are expected to have nearly equal dipole potentials in bilayers, while those of rarer lipid species vary considerably (Smaby and Brockman, 1990; Lairion and Disalvo, 2004). Thus, it would be of interest to thoroughly study the dipole potential in bilayers of different compositions to check whether this expectation holds. The dipole potential of sphingomyelins is considerably lower than that of phosphocholines. However, sphingomyelin (SM) in the plasma membrane is suggested to be present mostly in cholesterol-rich domains or as condensed complexes in SM:CHOL=1:1 ratio (see Epand, 2003), and for SM:CHOL=1:1 mixtures the dipole potential is approximately equal to the dipole potential of phosphatidylcholines (McIntosh et al., 1992). Thus, somewhat surprisingly, the major plasma membrane constituents studied this far have nearly equal dipole potentials as long as there is no dramatic difference in their acyl chains. Accordingly, the dipole potentials in plasma membranes could be nearly symmetrical. Considering the equal dipole potentials of PC and SM:CHOL=1:1 on monolayers, one may note that dipole potential is a major factor attributing to stability of domains in lipid films. The repulsive dipole pressure along with interfacial tension controls the shapes of domains in monolayers (Koker and McConnell, 1993; Lee and McConnell, 1993), and while the force due to dipoles in the mono-/bilayer

plane is attractive, there is a repulsive force associated to the vertical dipoles that contribute to dipole potential (Wohlert and Edholm, 2004). Differences between domains are seen, as in single component monolayers the dipole potential for fluid phospholipid domains is approx. 50–100 mV below that for solid domains (Heckl et al., 1989; Inoue and Yokoyama, 1994).

Whatever the role of dipole potential in cells, if any, dipole potential has been successfully employed in one application. The mammalian system for reception of taste consists of families of proteins, G protein coupled receptors, metabotropic glutamate receptors, and $GABA_B$ receptors (Lindemann, 2001; Scott, 2004). However, dipole potential and other membrane properties respond to substances of all basic tastes, sweet, bitter, salty, sour, and umami, to the extent that it allows for the construction of lipid-covered polymer membrane sensor, which uses responses of different lipid compositions to measure taste, and this sensor fatefully reproduces taste results of human test groups (Naito et al., 1993; Toko, 1998). Accordingly, similar to anaesthetics, it could suggest that either lipid/protein interactions are of importance or that at least the interactions of these compounds with bilayers reflect the properties of the binding sites on proteins.

6. Anaesthesia – the search for a mechanism

Freshmen medical students are almost always baffled by the fact that the primary target(s) of anaesthetics has remained elusive despite the almost 160-year history of anaesthesia beginning from Morton's public demonstration of ether anaesthesia in 1846 (see Antkowiak, 2001), or likely a more than 8 000 year history of alcohol-induced anaesthesia as a side-effect of alcohol consumption, brewing being invented no later than seventh millenium B.C. (McGovern et al., 2004). Almost as surprising is that there is no generally accepted definition of anaesthesia, though in the recent years it has become obvious that anaesthesia consists of a variety of more or less separate features such as antinociception, immobility, the inability to form memories, or amnesia, and the loss of conciousness or hypnosis (Antkowiak, 2001; Urban, 2002). During the past decades the mechanism of anaesthesia has been an active field of study, with journals dedicated solely to the mechanism of anaesthesia, not to mention the articles in the more general journals of anaesthesia, anaesthesiology, neurobiology, and biochemistry. Accordingly, it is impossible to review the whole field here, and I have largely relied on earlier reviews for background information not directly connected to this work.

6.1. Anaesthesia is heterogeneous at many levels

It has been only recently recognized that the phenomenon we usually call anaesthesia not only consists of many different parts such as antinociception, immobility, amnesia and hypnosis but that these separate phenomena emerge at different concentrations of a given anaesthetic, and that the sequence in which they emerge is different for different anaesthetics (Antkowiak, 2001; Urban, 2002). These phenomena are also related to changes in different regions of the nervous system, immobility and antinociception are produced mainly at the level of spinal chord in mammals, amnesia and hypnosis at the level of brain cortex. For the latter we must note that, unfortunately, memory and

consciousness themselves are practically unknown processes. Therefore it is difficult if not impossible to pinpoint the chain of events leading to loss of these properties.

This heterogenousness extends to lower levels, the neuronal networks being affected at many levels (Antognini and Carstens, 1998; Antkowiak, 2001; Århem et al., 2003; Urban, 2002) with various anaesthetics having effects on neurotransmitter release, receptor activation, dendritic and somatic signal integration and axonal conductance. Underlying these effects is a heterogeneity of action at an even lower, molecular level. Volatile anesthetics of the group halothane, isoflurane, sevoflurane etc. have perhaps the widest range of effects at clinically relevant concentrations, as they decrease transmitter

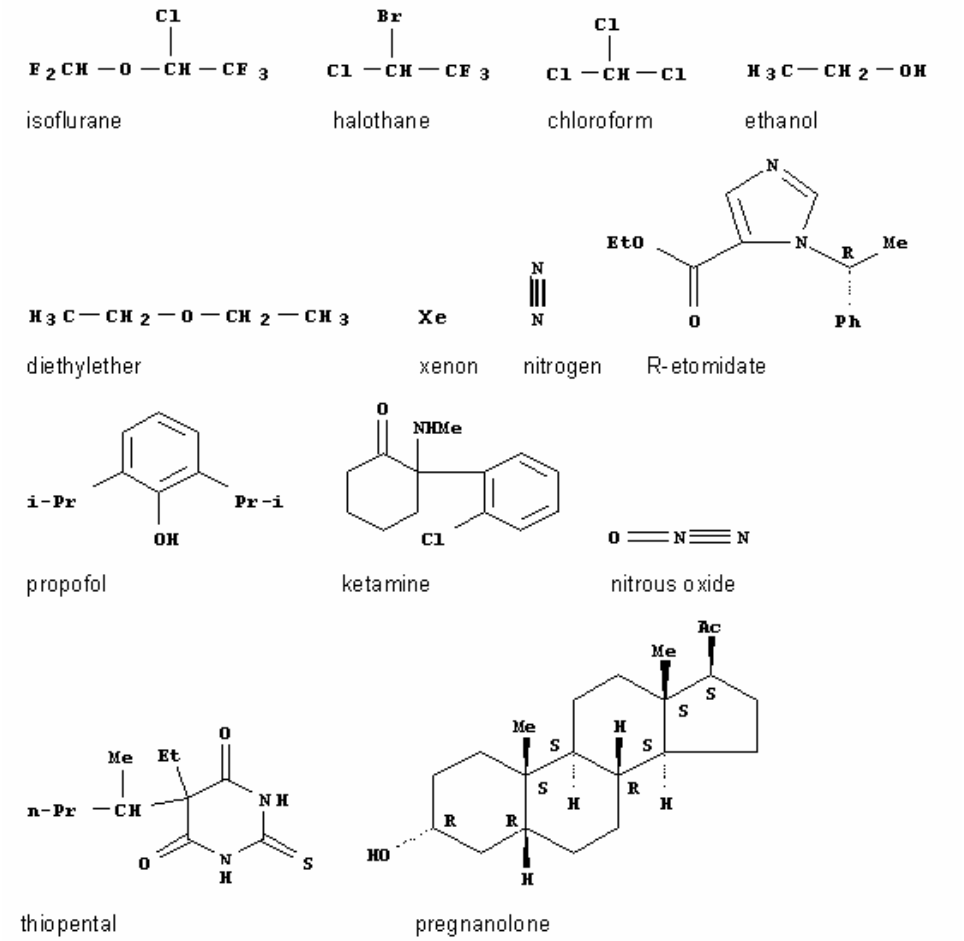

Figure I.6.1. Structures of various anaesthetics. Chiral carbons are shown and marked by R and S according to absolute stereochemistry. Ac=acetyl, Et=ethyl, Me=methyl, Ph=phenyl, i-Pr=isopropyl, n-Pr=n-propyl.

release, increase activation of $GABA_A$ receptors (with no significant effects on NMDA or AMPA receptors), affect signal integration by activating K^+ channels and inactivating Na^+ channels, and decrease axonal conductance. Other examples include riluzole, that decreases transmitter release and axonal conduction, and affects signal integration by inactivating Na^+ channels without effects on K^+ channels. Riluzole is of interest because at clinically relevant concentrations it has no effect on the activation of $GABA_A$, AMPA, or NMDA receptors, and it has no known effect on any other receptors. Effects of xenon and ketamine in turn appear to be limited mostly to the inactivation of NMDA receptors without any activating effect on the $GABA_A$ receptor (see Antkowiak, 2001), or ketamine having even an inactivating effect on it. In addition, ketamine is known to inactivate voltage-gated K^+ channels (see Århem et al., 2003). Anaesthetics also have effects on other proteins such as protein kinase C (Santiago Gomez et al., 2003). Moreover, especially at concentrations somewhat larger than those clinically relevant, anaesthetics affect functions of a large number of proteins that are in no way relevant for anaesthesia – firefly luciferase, for instance (Antkowiak, 2001; Franks and Lieb, 1998; Urban, 2002). Considering the wide range of effects of anaesthetics on proteins and lipids as well, it is perhaps not surprising that anaesthetics affect significantly not only animals from single-celled to humans (Oliver et al, 1991), but bacteria and bacterial proteins as well (see e.g. Adey et al., 1975; Batai et al., 1999; Molliex et al., 1998). It is clear that binding to any single protein cannot explain this multitude of different effects and the number of proteins affected not only *in vivo* but also *in vitro.*

Considering the widely heterogenous effects of anaesthetics at the molecular level, and the wide range of organisms affected by anaesthetics, it is hardly surprising that the structural variety of anesthetics themselves is enormous (see Fig. I.6.1). What is truly surprising is that despite all this heterogeneity at so many levels the potency of an anaesthetic correlates very well with the olive oil / water partition coefficient over several orders of magnitude. This is the so-called Meyer-Overton rule.

6.2. Meyer-Overton rule and the molecular level hypotheses of anaesthetic action

Though numerous exceptions exist, one unifying property of agents having anaesthetic effects has been discovered: the anaesthetic potency is reflected by the olive oil / water partition coefficient, producing a straight line in the double logarithmic plot of the anaesthetic concentration producing half-maximal effects against the olive oil/water partition coefficient. Accordingly, it was suggested by Meyer and Overton independently in 1899 and 1901 that anaesthesia might result from the effects of anaesthetics on membrane properties (see Antkowiak, 2001). More recently, the Meyer-Overton rule has been redefined so that anaesthetics prefer the permittivity of the lipid/water and protein/water interfaces (Ueda and Yoshida, 1999). The best correlation is obtained with ε_r=10–11 but the value varies for different aspects of anaesthesia (see Miller, 2002). After the early lipid theories, the ubiquitousness in biochemistry of specific binding onto specific proteins lead to suggestions that anaesthetics would bind directly to a protein or proteins, whose altered function would lead to anaesthesia. Early findings of a direct anaesthetic effect, apparently by competitive inhibition on lipid-free luciferase, showed that proteins also provide binding environments similar to that of the lipid/water interface, and that lipids are not necessarily required for anaesthesia (Aday et al., 1975). Later, the same result of apparent competitive inhibition was obtained with unrelated

luciferase from another source, with a wider range of anaesthetics, and it was shown to correlate with the anaesthetic potency. This competitive inhibition of other proteins was suggested as a mechanim of anaesthesia (Franks and Lieb, 1984). As a result the idea of direct binding to proteins became generally accepted. Incidentally, it has been argued that the competitive inhibition of luciferase is only apparent due to a complex reaction mechanism. There is very clear experimental evidence that various anesthetics already at low concentrations do destabilize the native, luciferin-binding conformation of luciferase (Ueda et al., 1998). Of course, by increasing the substrate, the native conformation can be again stabilized, and apparently V_{max} is only minimally affected. If competitive inhibition has been ditched, it is in fact easier to comprehend that anaesthetics affect (almost) all proteins at variable concentrations. Accordingly, the Meyer–Overton rule has been suggested to indicate the nonspecific binding of anaesthetics to various substances providing a suitable local environment (Ueda and Yoshida, 1999). Nevertheless, the active site of firefly luciferase binds anaesthetics in saturating solutions of anaesthetics, and a relatively simply model of combined competitive and noncompetitive inhibition can explain inhibition (Franks et al., 1998). Yet, soaking preformed crystals in saturating solutions may not describe the situation in solution very well. The issue is still unresolved. However, one possibility is that anaesthetics act with all the sites of intermediate polarity, leading to a net loosening of the protein structure by means of preferential solvation, while the interactions with the active site would be responsible for most of the inhibitory effect. As already discussed, especially at higher concentrations than those clinically relevant, anaesthetics affect most proteins. Yet, it is certain that not all of these actions have relevance regarding anaesthesia, which is particularly evident in the case of firefly luciferase, for instance, whatever the mechanism of inhibition. Additionally, based on studies of model proteins not related to anaesthesia, e.g. 40 % of proteins have been suggested to have suitable cavities for binding of xenon, and 10–15 % of cavities are large enough to bind some other volatile anaesthetics such as halothane. Additionally, as discussed by Cafiso (1998) and Cantor (1998), there is also a possibility of lipid-mediated action on relevant proteins. Consequently, there is a quest for finding those targets most relevant for anaesthesia.

In this quest there are certain rules derived from common sense: the relevant targets should be affected at clinically relevant concentrations, more potent related anaesthetics should have stronger effects at similar concentrations, non-anaesthetic compounds should not have the same effects as anaesthetics have, and the reversal of anaesthesia by increased pressure should apply. Likewise, the effects of temperature changes should be in the same direction as are they for anaesthesia. Finally, the effect on targets should display the same enantiomeric specificity as anaesthesia itself (see e.g. Franks and Lieb, 1998). As already discussed, it appears that one of the most important targets of anaesthetics is the type A γ-amino butyric acid (GABA) receptor that is a chloride channel gated by its ligand GABA. Indeed, many if not most anaesthetics do increase the chloride currents by activating this $GABA_A$ receptor either directly or indirectly, and the magnitude of the effect on $GABA_A$ receptor correlates well with the anaesthetic potency (Jenkins et al., 1999; Dickinson et al., 2000), and also with the stereospecificities of etomidate (Tomlin et al., 1998), steroid anaesthetics (Covey et al., 2000), barbiturates (Tomlin et al., 1999), and volatile anaesthetics (Harris et al., 1994). Also the temperature effects on anaesthesia are compatible with effects on $GABA_AR$ (Jenkins et al., 1999), although this, or the opposite effect of temperature on the *binding* to lipid bilayers does not mean that it is incompatible with binding to lipid bilayers. Even the effects on

$GABA_AR$s have heterogeneity. $GABA_AR$ is a protein formed of five subunits, and there are seven subunit classes, α, β, γ, δ, ε, θ, and π. There are six different types of α subunits, four types of β, four of γ and of the rest there is only one of each type. Most $GABA_AR$ in brains contain two α, two β, and one γ subunit, most often γ_2. Different subunits are required for the response to different drugs, γ subunits are required for benzodiazepine-sensitivity, and particularly sensitive are the γ_2 subunits, whose most essential amino acids are at sites homologous to the sites of the GABA-binding pockets α subunits. The binding pocket appears to be formed mostly by Phe-77 and Met-130 of γ_2 and His-101 of α_1 (Wafford et al., 2004). For some anaesthetics the primary importance $GABA_AR$ is beautifully demonstrated by the insensitivity of mutant animals to anaesthesia, e.g. in the case of etomidate the mutation N265M in the β_3 subunit abolishes anaesthesia by etomidate while the mutation N265S in the β_2 subunit abolishes the sedative effects of etomidate in mice (for review see Wafford et al., 2004). While no anaesthetic-insensitive mutant vertebrates have been reported in the case of volatile anaesthetics and alcohol, the sites most important for the sensitivity of $GABA_AR$ have been pinned down. A stretch of 45 amino acids mediatrs the action, with amino acids S270 and A291 being the most important for anaesthetic action, mutations S270I and A291W render $GABA_AR$ insensitive to ethanol and volatile anaesthetics, but the receptor is still sensitive to GABA and intravenous anaesthetic propofol (Mihic et al., 1997). The same region is involved in effects of etomidate, N290S mutation reduces both the etomidate-induced chloride current and benzodiazepine binding, while the ability of etomidate to augment GABA-induced chloride currents is retained (Moody, 1998). These sites are located at the transmembrane segments 2 and 3 at the protein/lipid/water interface (Mihic et al., 1997; Franks and Lieb, 1997). The effect of ethanol on $GABA_AR$ is very complex indeed. As reviewed by Mihic (1999), in some reports ethanol is found to have an effect on $GABA_AR$, while in others it is not found to have effect. This appears to depend not only on the subunit composition, for which presence of both δ and β_3 are essential (see Wallner et al., 2003; Harris and Mihic, 2004), and types of splice variants in $GABA_AR$, but also on the phosphorylation of $GABA_AR$ by protein kinase C (PKC), as well as on the intact contacts of $GABA_AR$ with the neuronal cytoskeleton (Mihic, 1999). Interestingly, but perhaps not surprisingly, volatile anaesthetics potentiate PKC by their effects on the regulatory domain which contains phospholipid and diacylglycerol binding sites (for reviews see Hemmings, 1998; Santiago Gomez et al., 2003).

The same interfacial site of transmembrane segments 2 and 3 appears to be essential not only for ethanol and volatile anaesthetics effects on $GABA_AR$ but on other ligand-gated ion channels of the cys-loop superfamily (for review see Dilger, 2002), such as inhibitory glycine receptors (Mihic et al., 1997) and serotonin receptors (Lopreato et al., 2004). Based on the crystal structure of the nicotinic acetylcholine receptor (another member of cys-loop superfamily) obtained at 4.0 Å resolution (Miyazawa et al., 2003), and the comparison of the sequences, the relevant residues for volatile anaesthetic action are located at the protein/lipid/water interface (Lopreato et al., 2004). Accordingly, direct interaction with receptor proteins appears likely. Interestingly, the size of a suitable cavity cannot be the only determinant of binding, as $GABA_AR$ is not affected by xenon or cyclopropane. Instead, smaller xenon affects NMDA receptors, has little effect on AMPA/kainate receptors, while larger isoflurane has a major effect on $GABA_AR$, and small but significant effect on both NMDA and AMPA/kainate receptors (see Franks et al., 1998; Miller, 2002; Sousa et al., 2000). Additionally, many of the experiments done

with $GABA_AR$ have been done by expressing mammalian receptor in *Xenopus laevis* oocytes, suggesting that if the binding to $GABA_AR$ site is not direct, the indirect interaction is conserved within vertebrates, at least. In the case of the photoactivatable derivative of etomidate, azietomidate, which by itself is a potent anesthetic, it has been possible to label several residues of nAChR, while $GABA_AR$ is present in so minute amounts, that the labelling of it has not been attempted (Hussain et al., 2003; Ziebell et al., 2004). Interestingly, genetic manipulation of *Caenohabditis elegans* has revealed that strains lacking $GABA_AR$ completely or enzymes for GABA synthesis both display normal sensitivity to volatile anaesthetics, raising questions about the causality between the detected correlations of actions of anaesthetics on $GABA_AR$ and anaesthesia (Swinderen et al., 1998). It has also been possible to produce both strains resistant and strains hypersensitive to the action of volatile anaesthetics, although different volatile anaesthetics have been found to display different profiles (Swinderen et al., 1998; Kayser et al., 1998). In *C. elegans* the mutations resulting in increased excitatory neurotransmitter release are among those responsible for the resistance to effects of volatile anaesthetics, and the mutation analysis has suggested that the primary target of volatile anaesthetics in *C. elegans* is a protein that interacts with the presynaptic protein syntaxin, whose function is to mediate fusion of neurotransmitter vesicles with the presynaptic plasmamembrane (Hawasli et al., 2004). Recently it was reported that also in the mammalian brain volatile anaesthetics inhibit exocytosis of presynaptic vesicles (Hemmings et al., 2005). Additionally, mutations in the large-conductance Ca^{2+}-activated K^+ channel (i.e. BK channel) can make the worm resistant to ethanol. Normally this channel is related to the control of excitatory neurotransmitter release, and data show that ethanol potentiates channel function, leading to a diminished release of excitatory neurotransmitters (Crowder, 2004). Curiously, phloretin also activates BK channels (Kraft et al., 2003). With respect to the applicability of *C. elegans* as a model we may note that both ethanol and volatile anaesthetics affect C. *elegans* at similar concentrations compared to vertebrates, including humans (Crowder et al., 1996; Crowder, 2004). It may also be noted that similarly to mammals the primary target of N_2O in *C. elegans* is the NMDA receptor (Nagele et al., 2004). Yet, *C. elegans* data may not affect the conclusions regarding the role of $GABA_AR$ in analgesia or amnesia, since, firstly, immobility is usually the parameter utilized in estimating anaesthetic potency in *C. elegans* and, secondly, neither immobility nor amnesia appear to be related to effects on $GABA_AR$ in mammals (Sonner et al., 2003; Liao et al., 2005). One complication in solving the action of anaesthetics is that anaesthesia is, like consciousness, a phenomenon reflecting the functional status of neuronal networks, not necessary directly related to the activity of a single type of a protein. In principle many molecular level events could lead to a similar effect at the level of neuronal networks (Urban, 2002; Århem et al., 2003).

Although it is by all means far beyond a reasonable doubt that specific proteins are of vital importance for the actions of anaesthetics, some suggest that the actions on these proteins are mediated by lipid membranes (Cafiso, 1998; Cantor, 1997a; 1997b; 1998) or by a simple physical mechanism such as change in hydration of lipids and proteins (Ueda and Yoshida, 1999). In recent years it has also been suggested that anaesthetics would inhibit exocytosis of presynaptic vesicles by their effects on the phase transition of phosphatidylserine (Kharakoz, 2001). Alternate mechanisms of lipid-mediated action include the effects of anaesthetics on dipole potential and spontaneous curvature (Cafiso, 1998), the latter of which is included in the more elegant hypothesis of the lateral pressure profile (Cantor, 1997a; 1997b; 1998) that has also spawned a far-reaching

hypothesis about the possible physiological analogue or endogenous form of anaesthesia (Cantor, 2003). Typically, also these properties show a good correlation with the efficiency of volatile anaesthetics (Kharakoz, 2001; Cafiso, 1998). Often lipid theories are criticized based on obviously faulty arguments. These include e.g. the claim that lipid theories offer no mechanistic link to anaesthetic action on the protein, that lipid-mediated actions could not explain the cut-off of the anaesthetic potency of alkanols at the length of approx. 12 carbons, that lipid theories cannot explain the different actions of true anaesthetics and nonimmobilizers, and that deviations from the Meyer-Overton series cannot be explained by lipid theories. Firstly, the effect of anaesthetics on dipole potential and lateral pressure profile, and their effect on a protein provides just as clear mechanistic link as does binding of anaesthetics directly to proteins. Secondly, not only can the cut-off be explained, but it is either theoretically predicted or experimentally verified both in case of lateral pressure profile (Cantor, 2001b) and dipole potential (Haydon and Elliot, 1986). Also without any detailed arguments it is easy to realize that once an alkanol is long enough, it will behave just as an another acyl chain, fitting well into bilayer. In addition to alkanols other series or compounds show cut-offs, e.g. very short alkanes have cut-offs much smaller than alkanols, fluorinated alkanols at even shorter lengths and for α,ω-diol-alkanes the cut-off is longer. All of these can be explained by the lateral pressure profile (Cantor, 2001a). Simulations have also shown that while anaesthetics both cause a significant change in dipole potential and associate to lipid/water interface, nonimmobilizers such as hexafluoroethane partition into the core of the bilayer, having an insignificant effect on dipole potential, and little effect on acyl chain order (Koubi et al., 2001; 2003). Accordingly, deviations from the Meyer-Overton series are predicted by lipid theories. If the size of the binding site in the target protein is thought to be the limiting factor it is less evident why smallest anaesthetic xenon does not affect all the target proteins, and why the cut-off size between the series should vary in a size independent manner. Synthetic polyhydric alkanols that are considerably larger (18 carbons and 4 hydroxyl groups) than the alkanol cut-off also show anaesthetic activity, as expected based on the lateral pressure profile hypothesis (Mohr et al., 2005). Yet, it is more difficult to explain their effect by the size of binding cavities. Nevertheless, there is evidence that binding to ligand-gated ion channels depends on size and hydropathy of amino acids at certain sites in the protein/lipid/water interface (Wick et al., 1998; Yamakura et al., 1999). Yet, as the authors discuss it is difficult to make a difference between the binding and transduction effects (Yamakura et al., 1999). Some evidence exists to favour the latter alternative: the mutations produce an opposite correlation for the binding of the actual ligands at unrelated sites. Furthermore, one should keep in mind that the lateral pressure profile can be changed in a very specific manner by compounds (see Patra, 2005; Carrillo-Tripp and Feller, 2005). Nevertheless, contradictory evidence is given by anaesthetic thiol or methylsulfonate analogues of alcohols and cysteine mutants of $GABA_AR$, as these compounds label certain interfacial amino acids that are located in preformed cavities (Mascia et al., 2000). However, labelling of interfacial amino acids would be expected in the framework of lipid-mediated hypotheses as well.

Scattered evidence exists for both direct protein binding and lipid-mediated actions. Nevertheless, what appears evident based on the above information is that finally, anaesthesia is mediated by only certain, relatively few specific proteins rather than having a generic effect on very many proteins. Some evidence, such as work by Tapia et al. (1998) appears to favour direct binding, as ethanol affects the glycine receptor already at concentrations which produce no effect on lipid phase. Yet, they did/could not measure

the properties most relevant for the lipid-based theories. On the other hand, alcohols and ketamine are able to inhibit acetylcholinesterase, alcohols apparently competitively and ketamine noncompetitively, only when this enzyme is bound either to native membranes or phospholipid vesicles (Curatola et al., 1979). However, this does not necessarily support lipid-mediated action, as the conformations of many proteins change upon association to membranes and cavities could be present only in a membrane-bound enzyme. In addition to the already discussed alternatives many other theories have been put forward, one suggesting that water and hydration plays a key role. Water appears to be required for interactions of biomolecules with anaesthetics (Shibata, 2002), and the hydration of model peptides, proteins, and membranes is affected by anaesthetics of different classes (see Tatara and Ueda, 2000, and references therein; Gattoni and Boffi, 2003). As there are transient lipid phase changes coupled to the travelling action potential, with the most recent hypothesis suggesting soliton propagation as an explanation (Heimburg and Jackson, 2005), it has also been suggested that the effects on lipid phase behaviour could hinder the action potentials (Ueda and Yoshida, 1999). Nonspecific solvating actions on proteins nevertheless appear to be different, or rather, modulated by actual binding. While halothane destabilizes myoglobin and lysozyme, it stabilizes albumins, which have cavities for binding of hydrophobic compounds (Tanner et al., 1998; see also Bhattacharya et al., 2000); the energetics of binding mostly derives from the release of hydration water from the protein cavity and from anaesthetic upon binding (Sawas et al., 2004). One suggestion made without a clear mechanistic connection is that the effects of anaesthetics could be mediated by changes in the lateral heterogeneity of membranes, as this is affected by anaesthetics and the distribution of some proteins in membrane domains can be altered by ethanol (Janes et al., 1992; Balasubramanian et al., 2002; Dai et al., 2005).

One piece of evidence that appears to favour direct binding to proteins is the enantioselectivity of anaesthetics. The effects on $GABA_AR$ reflect very well the observed enantioselectivity of anaesthesia (e.g. Moody et al., 1993; Tomlin et al., 1998; Covey et al., 2000; Dickinson et al., 2000). For volatile anaesthetic enantiomers the partition into several types of lipid matrices is identical (Dickinson et al., 1994), and effects of etomidate enantiomers on the phase behaviour of PCs show no differences (Tomlin, 1998). Therefore it appears more likely that direct interactions with proteins come to question, rather than indirect effects mediated by the lipid bilayer. A *caveat* is that the effects on properties suggested to be important for anaesthesia were not studied, additionally, only a few different phospholipids have been used. At this point we must point out to the reader that the lack of difference in interactions is not obvious, for phospholipids and cholesterol are chiral molecules, and not only they are capable of, but they also do display enantiospecific interactions (see e.g. Iwamoto et al., 2002; Pathirana et al., 1992). Interestingly, two different aspects appear to again couple the anaesthetics to membranes, although not to the indirect action. Firstly, the suggestion that neurosteroids and fatty acid amides that normally accumulate in sleep deprivation, might be endogenous ligands for anaesthetic recognition sites (Laws et al., 2001). Interestingly, cholesterol can counteract both the effects of such compounds on $GABA_AR$ as well as by itself cause changes into the opposite direction, i.e., decrease $GABA_AR$ currents (Sooksawate and Simmonds, 1998: 2001). Simulations and experiments with the simple ion channel gramicidin suggest that anaesthetics act on it to increase Na^+ conductance by disrupting the gramicidin-lipid interactions and by replacing the lipids. In comparison, nonimmobilizers do not have similar effects (Tang et al., 1999; Tang and Xu, 2002).

Preformed hydrophobic cavities played no role in this action. Integral membrane proteins generally appear to loosely bind several lipid molecules. This notion is based on various lines of evidence such as protein crystal structures that containing lipids that have nonnative conformations (Marsh and Páli, 2004). Lipids are known to affect the functioning of proteins. Accordingly, a case has been made that the lipid/protein interface could be the target of anaesthetics, anaesthetics being able to displace lipids from some of those sites (Altschuh et al., 2005; Pflugmacher and Sandermann, 1998; Walcher et al., 2001). Incidentally, we may note that likely the interaction of protein with polar lipid headgroups makes a significant contribution to total energetics, which would explain that the anaesthetic efficiency appears to increase with an increase in partioning of the anaesthetic to the regions of polarity of lipids headgroups.

6.3. Pregnanolone and steroid anaesthetics

It has been known for a long time that many steroids, including steroid hormones, are potent anaesthetics (see e.g. Atkinson et al., 1965; Duval et al., 1983). While other clearly nongenomic actions (such as those seen in erythrocytes devoid of a nucleus) were already known, these actions were largely forgotten after the discovery of the nuclear and cytoplasmic steroid receptors that act as transcription factors. A renewed interest can be seen in the 1990s, when the nongenomic effects again became acknowledged, and it became obvious that several if not most steroids have rapid actions that are not related to changes in gene expression (see e.g. Aitken et al., 1996; Bandyopadhyay et al., 1998; Christ et al., 1995; Hervé et al., 1996; Koukoutari et al., 1997; Mendoza et al., 1995; Morley et al., 1992; Morrill et al., 1998; Wehling, 1997). One of the main locations of nongenomic steroid activity appears to be the brain (Joëls, 1997; McEwen et al., 1990; Ramirez et al., 1990), steroids affecting directly $GABA_AR$ (Lambert et al., 1990) and the nicotinic acetylcholine receptor (Valera et al., 1992; Blanton et al., 1999). Steroids found to modulate the activity of neurons were dubbed neurosteroids, and these include many metabolites of steroid hormones. For example, neurosteroids pregnanolone and allopregnanolone are naturally occurring metabolites of progesterone, and like progesterone they also act as anaesthetics (see Zhu et al., 2001, and references therein).

The same problem that concerns other anaesthetics applies to steroid anaesthetics as well. The anaesthetic effects of steroids, even those structurally closely related, appear to correlate rather well with their effects on lipid bilayers, simple polypeptides and model proteins, as well as the hydration of these molecules (Makriyannis and Fesik, 1980; Makriyannis and Fesik, 1983; Makriyannis et al., 1986; Makriyannis et al., 1990; Makriyannis et al., 1991; Ueda et al., 1994; Mavromoustakos et al., 1995; Tatara and Ueda, 2000). For example, different anaesthetic potencies are found for a series of steroids whose structures differ by combinations of the isomerisms of the 3' hydroxyl group and the 5' hydrogen and the replacement of 11' keto group by two hydrogens. The anaesthetic potency for these steroids correlates well with the effects on phospholipid NMR spectra (Makriyannis et al., 1991). Considering this correlation, the Meyer–Overton rule appears to extend its grip over several different groups of anaesthetics, and the different suggested target sites on proteins, it appears feasible to follow thoughts of Ueda and co-workers (Ueda et al., 1994; Ueda and Yoshida, 1999). They propose that it is the non-specific tendency of an anaesthetic to be excluded from water, which drives its association with hydrophobic parts or patches on proteins and lipids.

The effects of steroids on $GABA_AR$ have also received considerable attention. Neurosteroids have effects on $GABA_AR$, and their effects are modulated by cholesterol (Sookswate and Simmonds, 1998; 2001). Due to its abundant availability, the nicotinic acetylcholine receptor of the same cys-loop superfamily of ligand-gated ion channels has often been used as a model ligand-gated ion channel. This protein contains several binding sites for lipids, and its activity is very strongly modulated by surrounding lipids, including anionic lipids and cholesterol (Barrantes, 2004). Its functions are modulated by steroids, and it has been suggested that the replacement of bound cholesterol and/or phospholipids by steroids is the mechanism for steroid effects (Blanton et al., 1999). Yet, the strength of binding and whether it actually is binding or mere preference of lipids to integral membrane proteins remains a matter of debate. If there is a specific binding of steroids to $GABA_AR$, it has been suggested to require several binding sites on $GABA_AR$ (Prince and Simmonds, 1993). However, attempts to demonstrate the actual binding have not yielded results. Despite good results in many cases, photolabelling of $GABA_AR$ has proved to be non-productive. Attempts to photolabel $GABA_AR$ with a $GABA_AR$-modulating steroid azipregnanolone could not demonstrate binding to $GABA_AR$. Instead, steroid labelled voltage-dependent anion channels (VDACs) that were found to coimmunoprecipitate with $GABA_AR$ (Darbandi-Tonkabon et al., 2003). VDACs have also been found to be labelled by the anaesthetic azietomidate (Husain et al., 2003). Yet, further experimentation showed that VDACs are not necessary for the effect of steroids on $GABA_AR$ (Darbandi-Tonkabon et al., 2004). A very weak interaction of the steroid with $GABA_AR$ could perhaps explain the results. No matter how the interaction is mediated, there obviously should be amino acids that are important if not critical for the transduction of signal from the presence of a steroid to chloride currents of $GABA_AR$. The highly interesting work of Morris and Amin (2004) studied the related $GABA_C\rho_1Rs$ that are largely insensitive to anaesthetics other than neurosteroids. Critical to the neurosteroid action are I307 and W328 located in transmembrane segments 2 and 3, in a location similar to the sites of action of anaesthetics at other ligand-gated ion channels. 5β-neurosteroids that have bent ring structure, pregnanolone for instance, act as inhibitors of the channel. On the contrary, 5α-neurosteroids that have a more planar ring structure, allopregnanolone for instance, act as potentiators (see Morris and Amin, 2004, and references therein). Accordingly, this receptor likely plays no role in anaesthesia. Mutations in the amino acid 307 may turn β-neurosteroids into activators, while 5α-neurosteroids appear to remain as activators despite mutations. Nevertheless, concentrations that have a half-maximal effect were seen to not change even when an inhibitor was turned into a potentiator by mutation. Mutations giving aa-307 a positive or negative charge produced similar results with respect to each other. Obviously, as the author concluded, the data is clearly inconsistent with the binding of steroids to that site. Action of pregnanolone was complex, it acted as an inhibitor or activator not only depending on the mutation, but also on the concentration used. The mode was dependent on the hydrophobicity of amino acid 307. Accordingly, to explain the results, the authors suggested that the hydrophobicity affects the position taken by the amino acid at the lipid/water interface, and they further suggested that the amino acid acts as a sensor of the local polarity and pressure profile. This kind of action would also explain the mutation experiments with other ligand-gated ion channels, which indeed appear to be consistent with the interfacial hydration, polarity (Tatara and Ueda, 2000), dipole potential (Cafiso, 1998) and lateral pressure profile (Cantor, 1997a,b; 1998; 2001; 2004; 2005) suggestions

of anaesthetic action. Likewise, the suggestion does not disagree with results of experiments and simulations on the effects of anaesthetics on gramicidin (Tang et al., 1999; Tang and Xu, 2002).

Despite the good agreement of mutation data with suggestions of lipid-mediated anaesthesia, anaesthetic neurosteroids also show features discouraging for advocates of lipid-mediated anaesthesia. First, a report shows that while various animals from *Paramecia* of the phylum of single-celled *Protozoa* to vertebrates such as frogs and humans are sensitive to ether and alkanols, only vertebrates (or possibly all craniates, as hagfishes were not tested) show sensitivity to pregnanolone. Even in *Cephalochordata* (lancelets) and *Urochordata* (tunicates) there was no sensitivity (Oliver et al., 1991). Additionally, while no difference between enantiomers is seen for pregnanolone, the 5α-neurosteroid allopregnanolone as well as some other steroid anaesthetics show enantioselectivity in their anaesthetic effect as well as in their effect on $GABA_AR$ (see e.g. Covey et al., 2000). There is an interest to understand better the interactions of neurosteroids with bilayers as well as the effects of neurosteroids on lipid bilayers. This is particularly important, since neurosteroids have been shown to have slow-kinetic action on $GABA_AR$, which was suggested to be dependent on their accumulation in the bilayer reservoir (Shu et al., 2004).

PART II: AIMS OF THE STUDY

This study attempted to clarify some of the interactions taking place at the lipid-water interface, and find new fluorescence spectroscopic approaches to study the properties of the lipid/water interface.

First, we wanted to evaluate the effect of the dipole potential on the interactions of membranes with small molecules and ions.

Second, we aimed to elucidate other interfacial factors affecting the binding of drugs to lipid bilayers.

Third, we wanted to utilize stopped-flow fluorescence measurements of the reduction of NBD by dithionite at lipid phase transitions, allowing for the evaluation of interfacial changes during transition. Another goal was to better evaluate other factors contributing to the dipole potential related measurements.

Fourth, we aimed at finding out if organic kosmotropic compounds display compound-specific effects on the surface tension at the air/water interface, their mode of action, and if these effects correlate with those seen in lipid bilayers.

PART III: METHODS

1. Materials

5β-Pregnan-3α-ol-20-one (pregnanolone) was obtained from Steraloids (Wilton, NH, USA). 6-lauryl-2-dimethylaminonaphthalene (Laurdan), 6-propionyl-2-dimethylamino-naphthalene (Prodan) and 4-[2-[6-(dioctylamino)-2-naphthalenyl]ethenyl]-1-(3-sulfopropyl)-pyridinium (di-8-ANEPPS) were from Molecular Probes (Eugene, OR, USA), and 1,6-diphenyl-1,3,5-hexatriene (DPH) from EGA Chemie (Steinheim, Germany). NBD-labelled lipids were from Avanti Polar Lipids, and other chemicals from Sigma-Aldrich. Purity of the lipids was checked on silicic acid coated plates (Merck) using chloroform:methanol:water (65:25:4, v/v) as the solvent. Lipid stock solutions were made in chloroform. Concentrations of the non-fluorescent lipids were determined gravimetrically using a high precision Cahn 2000 electrobalance (Cahn Instruments, Inc., Cerritos, CA, USA). Concentrations of the fluorescent probes were determined photometrically using conditions and absorptivities provided by Molecular Probes.

2. Preparation of unilamellar vesicles

First, appropriate amounts of the lipid stock solutions were mixed, evaporated to dryness under a gentle nitrogen stream, and then maintained under reduced pressure for a minimum of one and a half hours to remove any residual solvent. The dry lipid residues were hydrated with buffer, whereafter the suspensions were extensively vortexed, incubated for 30 min at 50–60°C with continuous shaking, sonicated for 2 min with a bath sonicator, and incubated for another 30 min at 50–60°C. This produces a solution of multilamellar vesicles in case of bilayer-forming uncharged phospholipids (and in case of charged phospholipids in the presence of a sufficient salt concentration).

To produce unilamellar vesicles these solutions of multilamellar lipid vesicles were extruded with a LiposoFast pneumatic low-pressure homogenizer (Avestin, Ottawa, Canada) 19 times through Millipore (Bedford, MA, USA) polycarbonate filters (pore size 100 nm) at a temperature well above the main phase transition temperature of the lipid. This produces mostly unilamellar vesicles whose average number of lamellae and size distribution depends on the extrusion pore size, extrusion pressure and the number of times the vesicles are extruded through the pores (Berger et al., 2001; Patty and Frisken, 2003). After extrusion, the liposome solutions were kept at 4°C or on ice until used.

3. Surface tension and monolayer measurements

All the monolayer measurements were done with apparati manufactured by Kibron Inc (Helsinki, Finland). Typically, stock solutions of lipids in $CHCl_3$ were applied on the solution surface to create a lipid monolayer. After allowing for evaporation of $CHCl_3$, the surface tension/pressure was measured.

Two types of methods are commonly used in the measurement of surface tension of lipid monolayers: the Wilhelmy (Wilhelmy, 1863) and the du Noüy (du Noüy, 1927) type of methods, in which the mass of the meniscus is weighed (see Allan, 1958). The mass is determined by the volume and thus the shape of the meniscus. Both these methods, as well as other methods such as sessile drop, pendant drop, and capillary rise are based on single law attributed to Laplace:

$$\frac{1}{R_1}+\frac{1}{R_2}=\frac{\Delta P}{\gamma}$$

where R_1 and R_2 are the principal radii of curvature at a given point in the interface, ΔP is the pressure difference, and γ is the surface or interfacial tension, depending on the interface. (Occasionally the formula is presented in a form in which the mean curvature $H=(1/R_1+1/R_2)/2$ replaces the principal radii of curvature). This fundamental equation holds for any curved surface, and dictates the forms of menisci. When presented in the differential form and when only the properties of surface/interfacial tension and gravity are present, the equation relating the coordinates of the interface to the forces takes the form

$$\frac{d^2z/dx^2}{\left(1+\left(dz/dx\right)^2\right)^{3/2}}+\frac{dz/dx}{x\left(1+\left(dz/dx\right)^2\right)^{1/2}}=\frac{\Delta\rho g}{\gamma}z,$$

which, unfortunately, is not solvable analytically in three dimensions, and must be numerically solved. However, it is analytically solvable in two-dimensions, and the Wilhelmy plate method is based on the assumption that the plate is so thin and long, that the contribution of the menisci at the ends of the plate may be neglected, and that the situation may be approximated by the two-dimensional case. In the case of the du Noüy ring method numerical solutions have been calculated and experimentally verified (see e.g. Freud and Freud, 1930; Harkins and Jordan, 1930).

Particularly in our case, the instrument by Kibron uses a metal alloy rod with a circular cross-section, and this situation is essentially similar to du Noüy ring case (Padday and Pitt, 1972; Padday et al., 1975). The only exception is that the maximum force is not measured but instead the force on the probe partly immersed in the meniscus. The instrument measures the surface tension based on calibration by the weight of the meniscus of pure water. Data were collected and analysed using dedicated software (Filmware) provided by the instrument manufacturer.

Usually in lipid monolayer work the surface pressure is used instead of the surface tension. Surface pressure (π) is defined as the difference between the surface tensions of the pure (γ_0) and the surfactant-covered surfaces (γ),

$\pi = \gamma_0 - \gamma$.

There are several contributions to γ, and, as a result, π as well consists of multiple components (see Kinnunen and Alakoskela, manuscript in preparation, for more detailed discussion). Basically, there are the contracting (tension) components from the lipid/water and the air/lipid interfaces, and the expanding components from the pull of the free surface and interlipid repulsive forces.

Monolayer surface pressure as well as other monolayer measurements such as the surface potential have been widely used in the studies of lipid properties and the interactions between various compounds and lipids (see e.g. Kaganer et al., 1999).

3.1. Penetration experiments (OP IV)

As described, the surface pressure (π) was recorded with a metal alloy wire attached to a microbalance and used magnetic stir bars in circular wells (1.2 ml, ∅ 1 cm). The subphase was 5 mM Hepes, 0.1 mM EDTA, pH 7.4. The pregnanolone stock was dissolved in DMSO Phospholipids were dissolved in $CHCl_3$ (final concentration ~0.5 mM) and were applied onto the air-water interface by a microsyringe. The lipid films were allowed to equilibrate for approx. 10 min to reach a stable initial surface pressure (π_0). Subsequently, pregnanolone was injected to obtain a final pregnanolone concentration of 2 μM. Association of pregnanolone is seen as an increase in π. The difference between final π and π_0 was used to calculate the increment in surface pressure, $\Delta\pi$ ($\Delta\pi=\pi-\pi_0$). The results are presented as $\Delta\pi$ vs. π_0 (Brockman, 1999). The final concentration of DMSO was <1 v-%, which did not affect π. All measurements were done at ≈ 22 °C.

Compression isotherms were recorded on a trough with two moving barriers (μTroughS). The lipid mixtures (dissolved in $CHCl_3$) were again applied onto the air-water interface using a microsyringe. The obtained compression isotherms consisting of approx. 800–1000 points were smoothed by moving average of 11 points, which had no visible effect on the curve shape. The smoothed data set was used for the calculation of the isothermal area compressibility κ_T per chain according to the formula

$$\kappa_T = -\frac{1}{A_c}\left(\frac{\partial A_c}{\partial \pi}\right)_T$$

where A_c represents the area per chain. Linear interpolation was used to create data sets of 800 points with the corresponding κ_T and π values, and κ_T was then presented as a function of surface pressure π. With aid of the curves obtained using numerical integration from the initial π_0 (before the addition of pregnanolone into the subphase) to the final π_f (new equilibrium value for π after pregnanolone addition) was carried out in order to compute the apparent changes in area/chain for the monolayer constituents, ΔA_c,

$$\Delta A_c = \left[1-\exp\left(\int_{\pi_i}^{\pi_f} \kappa_T d\pi\right)\right] A_{ci}$$

where A_{ci} is the initial area per chain. The meaning of the obtained ΔA_c value was clarified for viewing purposes by computing the number of pregnanolone molecules penetrating into the monolayer for every 1000 chains using the surface area of 57 $Å^2$, obtained from the γ vs. concentration curve fitting by the automated CMC measurement instrument Delta-8 of Kibron Inc. In the calculation of this value pregnanolone is considered to be a hard, non-interacting particle that occupies the same area in the phospholipid monolayers as on a clean air-water interface. Yet, pregnanolone and phospholipids could very well have preferential interactions which are incompatible with the first assumption, and therefore the number of pregnanolone molecules penetrating

into the monolayer would be underestimated. Likewise, if the pregnanolone molecules reside in the phospholipid headgroup region as our results suggest, then it is possible that in the vertical profile across the monolayer there are acyl chains under the cover of pregnanolone, and accordingly, the effective area occupied by pregnanolone would be somewhat smaller than the area on the clean air-water interface. Again, the above calculations would slightly underestimate the number of pregnanolone molecules penetrating into the monolayer. These calculations thus give an estimate for the lower limit of pregnanolone molecules associating with the membrane. Importantly, these data further allow for a comparison of the penetration of pregnanolone into monolayers consisting of different lipids. Using this method previously for different film compositions, it was shown that the results for the penetration of estradiol and testosterone into monolayers agree qualitatively with those obtained for steroid association to liposomes assessed by capillary electrophoresis (Wiedmer et al., 2002).

3.2. Surface tension of osmolytes (OP III)

The effect of betaine, sucrose, and choline chloride on the surface tension at the air/solution interface was measured by a multichannel microtensiometer (MultiPi WS1, Kibron, Helsinki, Finland). All measurements were done at ambient temperature (22°C) and for each solute concentration at least six individual samples were measured.

4. Fluorescence spectroscopy

Fluorescence spectroscopy is a widely used tool in biochemistry and biophysics. Its strength and weakness lies in the use of probes. The fluorescent probes are strongly fluorescent, thus easily detectable, making fluorescence spectroscopy highly sensitive. Additionally, the interactions of probes with their environment are dependent on the nature of the probe, and the probe can partition/attach to different locations which can be utilized. At its best fluorescence spectroscopy utilizes nearly specific probes for obtaining data only on specific properties of probe surroundings. The weakness is that probes report only on the probe surroundings, and probes by their interactions with the surroundings also change the properties of the probe surroundings compared to those of bulk matrix. While this in most cases makes it difficult to make reliable, quantitative estimates on the properties, it does not prevent highly sensitive detection of changes in the properties of matrix, which is interacting with the immediate surroundings of the probe. While fluorescence spectroscopic measurements are simple, their interpretation is often difficult and requires detailed data about the characteristics of the fluorescent probe and its interactions.

In one of the works presented here, a novel application of NBD probes was introduced. To provide a basis for the discussion later in this study, I here briefly describe some basic aspects of fluorescence (for more complete introduction see e.g. Lakowicz, 1999, and Gilbert and Baggott, 1991).

4.1. Electronic or vibronic transitions

The basis of fluorescence spectroscopy is in the electronic transitions of molecules, which can be nicely presented in a Jabłoński diagram (Fig. III.4.1). At temperatures used in biological fluorescence spectroscopy the ground state molecules are typically almost exclusively at the lowest vibrational state, even if one includes those molecules that have been recently excited to higher energy states. As with typical illumination intensities only a very small fraction of molecules are excited at any given time. As the vast majority of dye molecules are in a singlet state in their lowest energy state, the ground state is presented as the singlet 0 or S_0 state, and the first excited electronic singlet state as S_1. The transition from the ground state to the excited state is coupled to absorption of a photon of appropriate energy. The movements of electrons are extremely fast compared to the movements of the more massive atomic nuclei, indeed, the Franck-Condon principle states that the movement of electrons to new positions can be considered complete before the nuclei respond to the change of state. The nuclei respond to the altered bonding properties only after the change in the electronic state is complete. Accordingly, the electronic transitions take place without change in the positions of the nuclei. The consequences of this vast timescale difference in response are of utmost importance for fluorescence.

First, let us consider absorption. When a photon is absorbed by a typical, structurally complex fluorophore and the molecule first becomes excited into the S_1 state, usually only a part of the molecules will end up in the lowest (ν=0) or even the second lowest (ν=1) vibrational energy state. Instead most molecules end up in some higher vibrational energy state, whose wave function has the best overlap with the ground state wave function. In layman's terms this could be rephrased so that for the process of absorption the nuclei remain in their ground state positions, and as the electronic excitation changes the bonding properties of the orbitals and thus the equilibrium positions of the nuclei, the molecule will mostly end up in the vibrational state of the electronic S_1 state in which the nuclei due to vibrational excitation are likely to be found in positions close to those of the S_0 vibrational ground state. The steps between the vibrational energy levels are small, and the energy can be easily given in collisions to e.g. solvent molecules, resulting in a rapid relaxation between the vibrational states. For the purposes of fluorescence spectroscopy we may consider vibrational relaxation (VR) instant. Accordingly, after excitation to the higher vibrational states of S_1 state, the molecules rapidly relax to the lowest vibrational energy level. Due to rapid internal conversion between higher electronic states and S_1, these higher electronic states also rapidly relax to the lowest vibrational energy level of S_1. Thus (practically) all fluorescence originates from the lowest vibrational energy state of the first excited electronic state. This principle called Kasha's rule is also highly relevant considering the new fluorescence applications developed in this work.

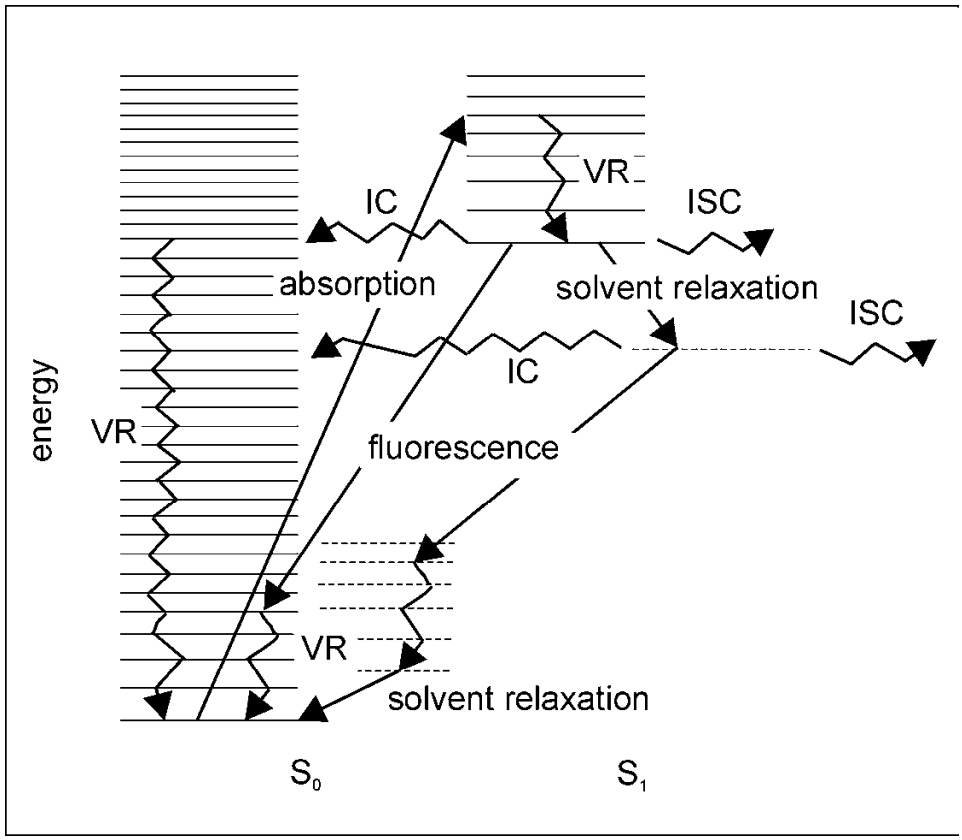

Figure III.4.1 A simplified Jabłoński diagram based mostly on Gilbert and Baggott (1991). The vertical axis corresponds to energy, the horizontal axis to different electronic states. VR indicates vibrational relaxation, IC indicates internal conversion between singlet states, and ISC is intersystem crossing between a singlet and a triplet state (not shown). Additional energy states related to the solvent relaxation are shown with broken lines. The energy differences related to solvent relaxation are considerably exaggerated for the sake of clarity.

The principle of overlap applies for the radiative relaxation process of fluorescence as well, and so the lowest vibrational state of S_1 typically relaxes to the higher vibrational states of the electronic ground state S_0. As can be seen from the Fig. III.4.1, the result of these processes is that the absorption energy change is larger than the fluorescence energy change, and correspondingly the wavelength for the emitted fluorescence is longer than that of absorption. This is further affected by interactions with solvents. Consider now that our S_0 vibrational ground state is also the state with optimal interactions with solvents, thus its energy is lower compared to those states where the solvent molecules surrounding the ground state molecule occasionally are in non-optimal orientations (one such state shown for S_0 with broken lines in Fig. III.4.1.). The distributions of electrons and even the positions of nuclei are altered following excitation into the S_1 state. Accordingly, the solvent interactions that were optimal for the S_0 state most likely are not optimal for S_1. The solvent nuclei are heavy as well and thus solvent reorientation and the process of optimizing interactions (except for fast polarization of the solvent electron cloud) is very slow compared to absorption and often slow compared to vibrational

relaxation (at least in cases relevant for this study). Accordingly, for the S_1 state there is solvent relaxation from the non-optimized state (solid lines) to the optimized state (broken line). Further, when the fast process of fluorescence occurs the relaxation now goes to the ground state with the solvent molecules having non-optimal interactions (broken lines for S_0), and is followed by another event of solvent relaxation. Consequently, the Stokes' shift, i.e. the difference between absorption and emission wavelengths (or energies), is larger in the presence of solvents, and furthermore, it is larger for solvents that interact more strongly with the fluorophore. Typically such strongly interacting solvents are either more polar, or capable of forming hydrogen bonds with the fluorophore.

The quantum yield of fluorescence, i.e. the ratio of photons emitted to photons absorbed is related to (neglecting the intersystem crossing) the relation of the radiative relaxation rate to the nonradiative rates. The latter in the absence of quenching and resonance energy transfer are mostly presented by the rate of internal conversion from S_1 to S_0, possibly via a triplet state, and in any case this is followed by a rapid vibrational relaxation to the lowest vibrational level of S_0. The fluorescence intensity recorded with constant illumination is affected not only by photon energies and quantum yield, but also by the probability of absorption for the fluorophore at the given conditions, a property which is usually presented by the molar absorptivity at a given wavelength and environment.

Of vital importance for anyone utilizing fluorescence spectroscopic measurements are the concepts of the dipole moment and especially the transition dipole moment. At the level required by a practicing bioscientist, most of the fluorescence spectroscopy can be easily understood and explained in terms of the transition dipole moments. The oscillating transition dipole interacts with the oscillating electric field of light. The oscillating transition dipole is capable of absorbing or emitting light if the frequencies of light and the dipole match, and in if in the case of emission molecule has enough energy for a photon. In fact, the total absorptivity and the rate of emission are related to the square of the respective transition dipole moment. These transition dipole moments should not be confused with the difference between the dipole moments of the excited and the ground states (Toptygin, 2003).

4.2. Fluorescence anisotropy

Fluorescence anisotropy is defined by the equation (Lakowicz, 1999):

$$r = \frac{I_{VV} - I_{VH}}{I_{VV} + 2I_{VH}},$$

where r is the fluorescence anisotropy, and I_{VV} is the intensity of emission with both the excitation and emission polarizers in vertical position, and I_{VH} is the intensity of emission with the excitation polarizer in the vertical and the emission polarizer in the horizontal position. In practical situations one has to correct for the different response of the detection system (largely due to gratings) to vertically and horizontally plane-polarized light (see Lakowicz, 1999). Fluorescence anisotropy can be easily understood by excluding the higher terms of multipole expansion and using the following simplified, schematic model where transitions are discussed only in terms of the absorption and emission (transition) dipole moments. In the transition a change in the amplitude (i.e.

energy) of an oscillating dipole (transition dipole) is coupled to the absorption or emission of a photon. The oscillations of the dipole field are along the dipole axis, and thus the polarization of the absorbed or the emitted photon is along the dipole axis, too. Thus, if the molecule is completely motionless and both its absorption and emission dipole moments are along the vertical z-axis, the polarization only will have the vertical component. If the absorption and emission dipole lie along the horizontal axes (x or y), only the horizontal polarization components will be present. In anisotropy experiments the vertical polarization of the exciting light selects for mainly (according to the square of dot product) vertically oriented absorption dipoles, and if the orientation of the probe changes during the lifetime of the excited state, then there will be decrease in the vertical polarization component and an increase in the horizontal polarization component. If the orientation was random originally, even for completely motionless molecules the maximum anisotropy is 0.4, as one can easily see by integrating the product of the probability of absorption ($\sim\cos^2\theta$) and the vertical component ($\cos^2\theta$) or x-axis component ($\cos^2\varphi\times\sin^2\theta$) in spherical coordinates over the angles. However, if due to vibrational relaxation of the excited state there is an angle between the absorption and the emission dipole moments, some change in anisotropy is expected also for stationary molecules, and even this value cannot be attained. Thus, in most practical cases (with non-oriented samples) the maximum anisotropy is less than 0.4. If the orientation of individual molecules changes with time, anisotropy will also decrease as a function of time. The steady-state anisotropy, used in the studies presented here, is the integral of the product of the time-dependent anisotropy and time-dependent (relative) intensity. The former is for a randomly oriented sample dependent on the rotational rate(s) and the angle between the emission and absorption dipoles, and the latter is dependent on the fluorescence lifetime.

4.3. Electrochromism and solvatochromism

In the electronic (or vibronic) transitions of a molecule the charge distribution of the molecule changes. Accordingly, it appears intuitively obvious that an electric field in the direction of charge movement should affect the energetics of the transition. Indeed, this is the case. Depending on the direction of the electric field the transition energy may increase (shift to shorter wavelengths) or decrease (shift to longer wavelengths). Obviously for a random orientational distribution this leads to an increase in the half-width of the absorption or emission band, whereas true shifts are seen when molecules are oriented with respect to the field. Such fields with a certain, determined directions with respect to the molecular axis, are not only present for attached molecules, but also spontaneously rise in polar solvents. In this case, the solvent dipoles surrounding the dye molecule become oriented due to interactions with the dipole of the dye. If one makes the gedanken experiment, and removes the dye from the center, in the cavity there is an electric field originating from the solvent dipoles. This field is called the reaction field, and it is one of the factors contributing to solvatochromism, i.e. the change in colour of the dye upon a change in solvent properties. Similarly, as both vibronic transitions and hydrogen bonds affect the electron probability distribution of the molecule, hydrogen bonding may cause a shift in the spectrum of a dye. Other solvent-originating factors causing shifts include factors such as different charge and multipole interactions, protonation, and viscosity, especially for dyes whose transition is followed by a large

rotation/reorientation of the molecular moieties with respect to each other (for references see e.g. Liptay, 1969; Suppan and Ghoneim, 1997; Lakowicz, 1999).

While the effects of electric fields and solvents on the absorption and emission band positions are relatively well-known and well described by simple models such as Lippert–Mataga (Lakowicz, 1999), much less is known about the effects of solvents on the absorptivity and the radiative relaxation rate of a dye. The mathematical as well as the conceptual description of these effects is much more complex compared to those of spectral shifts. These effects are not regular solvatochromism. One group of such effects originates from the cavity shape in solvents and the refractive index of the solvent, leading to strong effects on absorptivity and radiative relaxation rate (Toptygin, 2003).

4.4. Di-8-ANEPPS as a dipole potential indicator (OP IV)

Di-8-ANEPPS is a potential-sensitive dye, which has been characterized by Loew and coworkers. Most likely the response of this dye is of electrochromic origin, thus it reacts to all electrical fields imposed over the dye axis. However, the location of the dye appears to be such that changes in the membrane dipole potential evoke by far the strongest response in its fluorescence (Gross et al., 1994). The polarization of the dye in the ground state is affected by the surrounding electric field, and thus the ratio of the absorptivities of the absorption bands is altered. Gross et al. described a good correlation between the membrane dipole potential and the ratio R of dye fluorescence at the emission wavelength of 620 nm when excited at the two different wavelengths of 440 and 530 nm,

$$R_{620\ nm} = \frac{I_{440\ nm}}{I_{530\ nm}}.$$

The response of the dye has been studied to some extent, and the results suggest that contributions from confounding sources like dye-dye interaction can be further reduced if the emission intensity is recorded at 670 nm and $X_{dye} \leq 0.005$ (Clarke and Kane, 1997; Clarke 1997). Unfortunately, setting the detection at the red-edge of the emission spectrum at 670 nm results in considerable loss of intensity compared to the originally suggested detection wavelength of 620 nm that was also used in this study.

4.5. NBD fluorescence (OP I, II)

7-nitro-2,1,3-benzoxadiazol-4-yl (NBD) is a widely used fluorescent moiety in the study of biological systems (for review see Chattopadhyay, 1990). In studies on biological and model membranes NBD-labelled lipids have been employed in a wide range of applications including the visualisation of domain morphology by epifluorescence microscopy of lipid monolayers (Weis, 1991), the measurement of temperature in living cells (Chapman et al., 1995), detection of bilayer-to-hexagonal phase transition (Hong et al., 1988; Stubbs et al., 1989; Han and Gross, 1992), studies of organizational changes in membranes (Mukherjee and Chattopadhyay, 1996), and detection of interdigitation (Li and Kam, 1997). Some of the studies have simply utilized the partitioning of NBD-

labelled lipids between different lipid phases to detect these phases, while others have employed the fluorescence characteristics of the dye.

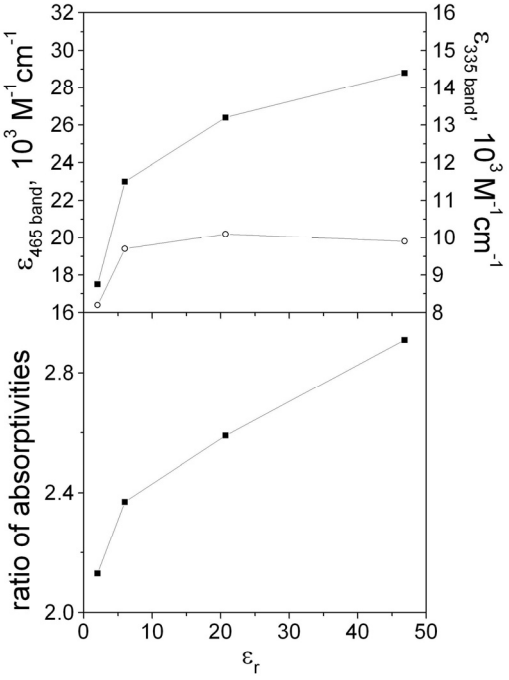

Fig. III.4.3. The molar absorptivities and the absorptivity ratio of NBD bands vs. relative permittivity of the solvent. In the upper panel the absorptivities of the 465 nm (filled squares) and 335 nm (open circles) bands are shown plotted against relative permittivity. The lower panel shows the ratio of these absorptivities, $\varepsilon_{465\ nm\ band}/\varepsilon_{335nm\ band}$, as a function of relative permittivity. The figure has been drawn based on data in Fery-Forgues et al., 1993.

Simple NBD derivatives have three major absorbance bands in the visible and near UV region, at approx. 420, 306–360 and 225 nm (Lancet and Pecht, 1977; Fery-Forgues et al., 1993). These bands are slightly shifted for phospholipid derivatives. The first band is observed near 465 nm (Fig. 1), and the second at approx. 335 nm. The band near 465 nm is due to an intramolecular charge-transfer (ICT) type of transition (Paprica et al., 1993; Fery-Forgues et al., 1993), which is associated with a large (≈ 4 Debye) change in the dipole moment (Mukherjee at al. 1994). The latter 335 nm band corresponds to an ordinary $\pi^*\leftarrow\pi$ transition (Fery-Forgues et al., 1993). Regardless of the absorbance band used for excitation, the maximum emission wavelength λ_{max} of NBD-labeled lipids lies at 520–535 nm (Fig. 1), as expected based on Kasha's rule. Absorbtivity, fluorescence intensity and both the absorbance maximum wavelength of the ICT transition and the corresponding emission maximum wavelength are sensitive to the polarity and the hydrogen bonding capability of the environment as well as to the presence of charge transfer donors (Lancet and Pecht, 1977; Fery-Forgues et al., 1993; Lin and Struve, 1991; Saha and Samanta, 1998). However, for the 335 nm absorbance band there is one difference: the absorptivity of this band is less strongly dependent on environmental polarity, which partly reflects environmental permittivity (see Fig. III.4.3).

An important feature of NBD is its strong self-quenching (Brown et al., 1994). In monolayers of pure NBD-labeled lipids strong hydrogen-bonding interactions between the NBD moieties can be detected (Tsukanova et al., 2002). The aggregation and self-quenching of NBD-acyl chain labelled lipid analogs in the gel phase is particularly

strong. While there is considerably less segregation and self-quenching for headgroup-labeled NBD-lipids, the presence of some segregation is supported by an analysis of the fluorescence lifetimes (Loura et al., 2000). The location of the NBD attached both to the headgroup and to the acyl-chain is within the interface in fluid phase lipids, as suggested by studies using the parallax method (Abrams and London, 1993; Chattopadhyay and London, 1987). Moreover, it appears that NBD attached via a 12 carbon spacer is located closer to the water phase than NBD attached to the headgroup, their distances from the membrane center being 19.8 and 18.9 Å, respectively.

4.6. Reduction of NBD by dithionite (OP I, II)

NBD-labeled lipids have been utilized also to study phospholipid asymmetry (McIntyre and Sleight, 1991). More specifically, when added to liposomes containing NBD-labeled lipids, either dithionite or its negatively charged radical form can rapidly reduce NBD to the nonfluorescent product, 7-amino-2,1,3-benzoxadiazol-4-yl (ABD, Fig. III.4.4), thus allowing for the assessment of the content of the probe in the outer leaflet of the bilayer. Simultaneously with this rapid reaction the dithionite ions diffuse slowly across the bilayer reducing the NBD groups in the inner leaflet also. The reduction of the NBD-labeled lipids by dithionite has further been used e.g. to make asymmetrically labeled liposomes (McIntyre and Sleight, 1991), to monitor lipid flip-flop (Williamson et al., 1995), and to study the properties of membranes undergoing phase transitions (Langner and Hui, 1993).

The kinetics of the reduction of NBD-labelled lipids by dithionite was measured using a stopped-flow spectrofluorometer (Olis RSM 1000F, On-Line Instruments, Inc., Bogart, GA, USA). In brief two parallel mounted syringes were pneumatically driven to inject the reactants into the rapid mixing quartz glass fluorescence observation chamber. One of the syringes was loaded with a liposome solution (with a total lipid concentration of 100 μM in 5.0 mM Hepes buffer, pH 7.4). The other syringe contained 10 mM dithionite in the buffer. The concentrations in the observation chamber were 5.0 mM for dithionite and 50 μM for lipid. The mole fraction X of the NBD-labelled lipids was 0.03 yielding a 1.5 μM concentration of the indicated fluorescent marker. The excitation wavelength of 470 nm was selected with a monochromator, and 21–62 spectra (473.5–626.5 nm) per second were measured.

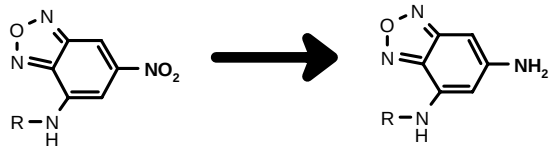

Figure III.4.4. Reduction of NBD by dithionite results in nonfluorescent ABD.

4.7. Laurdan and Prodan fluorescence (OP III, IV)

Laurdan and Prodan contain the same fluorescent moiety, having the basic structure of 6-acyl-(2-dimethylaminonaphthalene). For Prodan the acyl chain is a three carbon propionyl chain and for Laurdan a twelve carbon lauryl chain, making Prodan more water-soluble. The emission spectra of the probes typically consist of two main components, centred at approx. 440 and 490 nm. The blue-edge component is the prominent one in gel phase bilayers while the red-edge component is prominent in fluid bilayers. In the intermediate states the ratio of the two components is altered. The data is usually presented as GP values,

$$GP = \frac{I_{blue} - I_{red}}{I_{blue} + I_{red}} .$$

Studies have indicated, especially those using D_2O, that the spectral shift and thus also the GP value is related to the interaction between the interfacial water molecules and the probe moiety (Parasassi et al., 1998). Interestingly, for Laurdan one of the main determinants of probe hydration is the available space at the level of the lipid carbonyl groups where the probe is located. This is particularly useful, as Bagatolli et al. (1998) found that the Laurdan GP value is linearly correlated to the mean interbackbone distance for series of similar phospholipids (e.g. zwitterionic series, anionic series, glycolipid series etc.).

4.8. Emission anisotropy of DPH (OP IV)

One of the most widely used fluorescent probes in studies of biomembranes and their models is the highly fluorescent probe diphenylhexatriene (DPH) and its phospholipid derivatives, e.g. 2-(3-(diphenylhexatrienyl)propanoyl)-1-hexadecanoyl-*sn*-glycero-3-phosphocholine (DPH-PC). The DPH moiety is hydrophobic and rod-like, and is readily accommodated into phospholipid bilayers. The advantage that DPH probes offer is that their rotational motion and consequently emission anisotropy correlates well with the order of the surrounding phospholipid acyl chains. Accordingly, DPH has been used to study phospholipid phase transitions ever since the early days of membrane biophysics (Andrich and Vanderkooi, 1976). While most often the DPH fluorescence anisotropy is used only as a semiquantitative measure for fluorescence, it has been shown that the anisotropy ratios r_∞/r_0 available from time-resolved anisotropy measurements represent the square of the order parameter for DPH, and that these values agree very well with the order parameters from deuterium NMR for carbons 10–12 of DPPC acyl chains (Heyn, 1979).

The absorption maximum of DPH in membranes is approx. 354 nm, the emission maximum approx. 428 nm, and the fluorescence lifetime ≈10 ns (Lakowicz, 1999). In applications only anisotropy is ordinarily used. Yet, DPH is not insensitive to its environment. In fact, it is almost nonfluorescent in water which further acts to guarantee that most of the DPH emission originates from membrane bound DPH (Lakowicz, 1999). Additionally, the DPH fluorescence quantum yield in various solvents increases with increasing viscosity and solvent polarizability. A spectral red-shift accompanies increasing solvent polarizability (Dupuy and Montagu, 1997, and references therein).

The location, distribution, and orientation of DPH and DPH-labelled lipids in bilayers have been studied in detail, yet controversies exist. The analysis of the time-resolved fluorescence spectra of DPH in membranes has revealed a short-lived and red-shifted component, indicating that part of the probe resides in a relatively polar environment. This result was also supported by a more efficient energy transfer from the short-lived component to the fluorescein-labelled BSA (Konopasek et al., 1998, and references therein). According to depth-dependent quenching studies with DPH and its derivatives in a DOPC matrix, most of the fluorescing free DPH as well as the DPH moiety of DPH-PC are located deeply buried within the hydrophobic region. The distances from the bilayer centre for free DPH and the DPH moiety of DPH-PC are 7.8 and 6.9 Å, respectively. In DOPC matrix these values correspond to the levels of carbons 9 and 10, respectively, as calculated by Kaiser and London (1998; 1999). However, the parallax method of Kaiser and London only allows for detection of the average position, and not the distribution. The possible presence of two populations implies that the energy difference between these states should not be large, and that changes in the free energies for different DPH states in the membrane might lead to considerable differences in the occupation of the two states. Yet, the methods based on fluorescence quenching or spectral analysis detect only fluorescent populations, and independent data about the distribution is needed in order to detect also possible weakly fluorescing probe populations. A bimodal distribution of selectively deuterated DPH has been verified in neutron diffraction studies (Pebay-Peroula et al., 1994). More specifically, these authors calculated that in the $L_{\beta'}$ phase there are two DPH populations. The long axis of DPH for the first population is oriented parallel to the acyl chains, at a 30° angle to the bilayer normal, buried deep within the hydrophobic region. The long axis of DPH of the second, interfacial population, is oriented parallel to the membrane surface. In the L_{α} phase the first DPH population resides between the two leaflets and is parallel to the surface, with a wider distribution. However, a caveat regarding the study of Pebay-Peroula et al. is that in order to obtain a sufficient contrast they had to use X_{DPH}=0.1, whereas normally in fluorescence spectroscopy X_{DPH}=0.002 is used. As fluorescence quenching studies suggest another location that is not compatible with interleaflet population, it may be that the distribution of DPH in bilayers is a function of the DPH content of membranes. In a recent simulation study a simple, one-peaked DPH distribution was obtained. The DPH was in alignment with the acyl chains and was roughly at the level suggested by results of Kaiser and London (Repakova et al., 2004). Yet, the results of the simulation are cast in doubt, too. Firstly, the fluorescence spectroscopic studies by Konopasek et al. (1998) at low DPH mole fractions as well as the neutron diffraction of deuterated DPH (Pebay-Peroula et al., 1994) at high DPH mole fractions both suggest a presence of an interfacial DPH population. Secondly, a greatly simplified Monte Carlo simulations at low DPH mole fraction produces a DPH distribution agreeing with the results by Pebay-Peroula et al., although authors do not cite Pebay-Peroula work (Zandvoort et al., 1997). The lack of an interfacial population in MD simulations could simply derive from the fact that MD simulations do not take into account the molecular electronic polarizability, and thus they are indeed likely to make wrong predictions in situations where electronic polarizability is of importance. Such situations are likely to arise for nonpolar but highly polarizable particles, e.g. DPH or xenon, which according to experiments resides preferentially in the lipid/water interface (Xu and Tang, 1997), but partitions into tail region in MD simulations (Stimson, Vattulainen, Rog, and Karttunen, conference abstract, "Exploring the effect of anaesthetic gases on biomembranes",

http://cecam-workshop.memphys.sdu.dk/abstracts.shtml). The highly polarizable π electron ring structure of DPH is neglected in simulations, and the presence of an interfacial population of DPH thus appears possible despite the simulation results. Gratifyingly, no matter which hypothesis of distribution will turn out to be correct, it does not hinder the employment of DPH as a reporter of acyl chain order, as was done in our studies.

5. Osmolarity (OP III)

Osmotic activity for choline was measured by the freezing point depression method (Micro-Osmometer Model 3300, Advanced Instruments, Norwood, MA). All measurements were done in duplicate. Betaine and sucrose data were retrieved from the homepage of the Laboratory of Physical & Structural Biology at the National Institutes of Health (http://dir.nichd.nih.gov/Lpsb/docs/OsmoticStress.html), and this data had been measured by the vapour pressure method. Mostly the vapour pressure and the freezing point depression methods provide identical results, though the former suffers from the use of filter papers (Kiyosawa, 2003).

PART IV: RESULTS

1. Dipole potential affects the rate of NBD reduction by dithionite (OP I)

The negatively charged ion dithionite reduces the nitro group of NBD, resulting in a nonfluorescent compound. We studied the rate of this reaction, using NBD-labelled lipids embedded into different lipid matrices. We found that the simplest model yielding good agreement with the measured data was that of the two independent first-order reactions. Accordingly, the software provided by the instrument manufacturer was used to fit the data by the equation

$$U = A_1 e^{-k_1 t} + A_2 e^{-k_2 t},$$

where k_1 and k_2 represent the rate coefficients for the fast and slow components, respectively, A_1 and A_2 are the corresponding amplitudes, t represents time, and U is the photomultiplier tube output voltage. As the slower component is likely associated to the leakage of the dithionite through the bilayers and the flip-flop of lipids (McIntyre and Sleight, 1991; Langner and Hui, 1993), we concentrated our efforts on the fast reaction component. Of course, as the reaction is bimolecular, the kinetics is pseudo-first-order due to a much larger concentration of the dithionite anion remaining apparently constant for the whole time of the reaction. We found that in the case of the headgroup-labelled DPPN there is an approximately linear relationship between the rate of the faster reaction and the dipole potential changes produced either by including 6-KC or PHLOR into membranes or by changing DPPC to DHPC (Fig. IV.1.1). We could find a similar effect of the bilayer dipole potential on the rate of reduction of the NBD moiety of NBD-PC

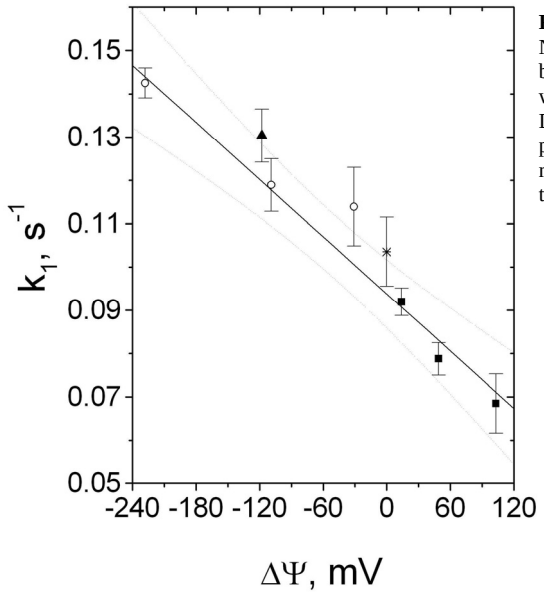

Figure IV.1.1. The rate of reduction of the NBD moiety of DPPN as a function of bilayer dipole potential at 50°C for vesicles with X_{DPPN}=0.03. The asterisk marks the pure DPPC bilayer matrix, open circles mark phloretin-containing samples, filled squares mark 6-KC-containing samples, and up triangle marks pure DHPC matrix.

(Fig. IV.1.2).

A striking observation is that the rate of reduction for NBD-PC is approx. fivefold compared to the rate of reduction of DPPN. The explanation for this is most likely the repulsion between the negative charges of the reducing agent dithionite and the DPPN. Nevertheless, the absolute effect of the dipole potential variation is similar for both DPPN and NBD-PC (Fig. IV.1.3).

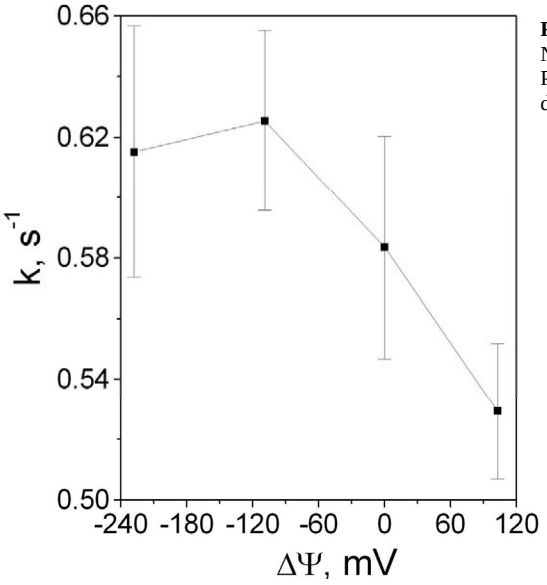

Figure IV.1.2. The rate of reduction of NBD moiety of NBD-PC. X_{NBD-PC}=0.03. PHLOR and 6-KC were used to vary dipole potential.

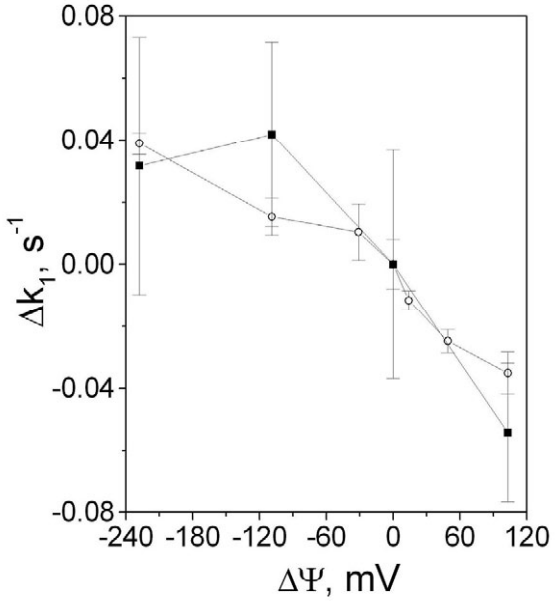

Figure IV.1.3. The difference in rate of reduction compared to pure DPPC matrix. Filled squares represent series for NBD-PC and open circles the series for DPPN.

Accordingly, the reduction rates suggest that for ions the penetration kinetics and thus the energetics for penetration to the level of NBD are greatly affected by the membrane dipole potential.

2. Fluorescence intensity of NBD is changed by dipole potential (OP I)

Not only is the rate of reduction affected by changes in the dipole potential for both DPPN (Fig. IV.2.1) and NBD-PC (Fig. IV.2.2) but the fluorescence intensities are also affected. Whereas the rate of reduction decreased with increasing dipole potential, the intensity of NBD-labelled lipids increases. For these as well as for the rate of reduction, the magnitude of change for both probes is relatively similar over the whole range. Obviously, the data suggests that dipole potential affects both the intensity and the reduction rate through a similar chain of events, such as changes in hydration, direct electrostatics, or change in probe orientation or location. However, as DHPC produced similar results despite a different hydration pressure (see section I.5.3) and as neither phloretin nor 6-ketocholestanol affect the water anisotropy deeper within the membrane, at the level of the carbonyl groups (Diaz et al., 1999), it seems that a more direct connection to dipole potential is likely, either by electrochromic effects or by change in probe location. Alternatively, the hydration pressure should not be connected to the orientation and the interactions of water molecules at the interface. The relationship to hydration is nevertheless favoured by more recent work with other neutral fluorescent probes (Duportail et al., 2001).

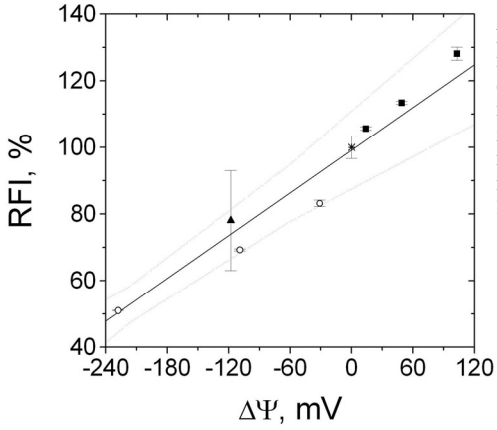

Figure IV.2.1. RFI of DPPN in matrices of different bilayer dipole potential. RFI represent maximum fluorescence intensity relative to that recorded from DPPN in DPPC matrix. Symbols as in Fig. IV.1.1. T=50°C, X_{DPPN}=0.03.

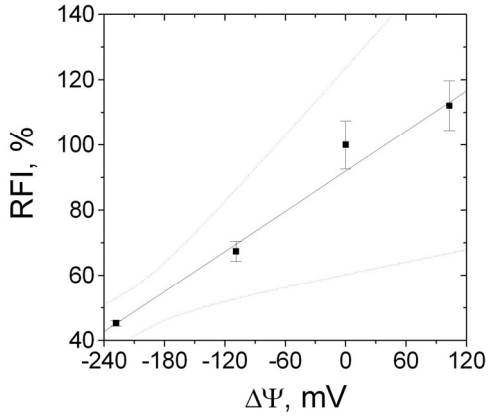

Figure IV.2.2. RFI of NBD-PC in matrices of different bilayer dipole potential. RFI represent maximum fluorescence intensity relative to that recorded from NBD-PC in DPPC matrix. T=50°C, X_{NBDPC}=0.03.

3. Redox reaction and NBD fluorescence as probes for phase transition (OP II)

In addition to studying the effects of dipole potential on the fluorescence of NBD-labelled lipid derivatives, we assessed the effects of phase transition on both NBD fluorescence characteristics and the rate of reduction by dithionite. As the partitioning of DPPN and NBD-PC into lipid domains is different, it is not surprising that the temperature-dependence of the reaction rate is also dissimilar.

As can be seen from figures IV.3.1 and IV.3.2, for DPPN the response to the phase transition is much stronger with fluorescence intensity being the exception. An easy interpretation for the behaviour of fluorescence intensity is instantly offered by the known self-quenching behaviour of the dye. Both DPPN and NBD-PC (Hong et al., 1988; Weis, 1991; Loura et al., 2000) are known to be enriched in domains in the gel phase matrix. However, for NBD-PC this segregation is extremely strong, with little partitioning into the gel phase, whereas for DPPN there is only minor segregation (Hong et al., 1988; Weis, 1991). In addition, the increase in intensity at the transition is known to vanish if sufficiently small mole fractions of the probe are used (Hong et al., 1988). Our intensity data agree perfectly with a decrease in self-quenching upon dispersion of the segregated probe into the bulk. One feature worth noticing in the intensity behaviour is that the ratio I_{470}/I_{335} of emission intensities of the probe excited at the ICT band (I_{470}) and the $\pi^*\leftarrow\pi$ band (I_{335}) wavelengths shows a minimum at the phase transition. As discussed in section III.4.5., this implies that the surroundings of the probe appear less polar to the probe. For NBD-PC this minimum is not found.

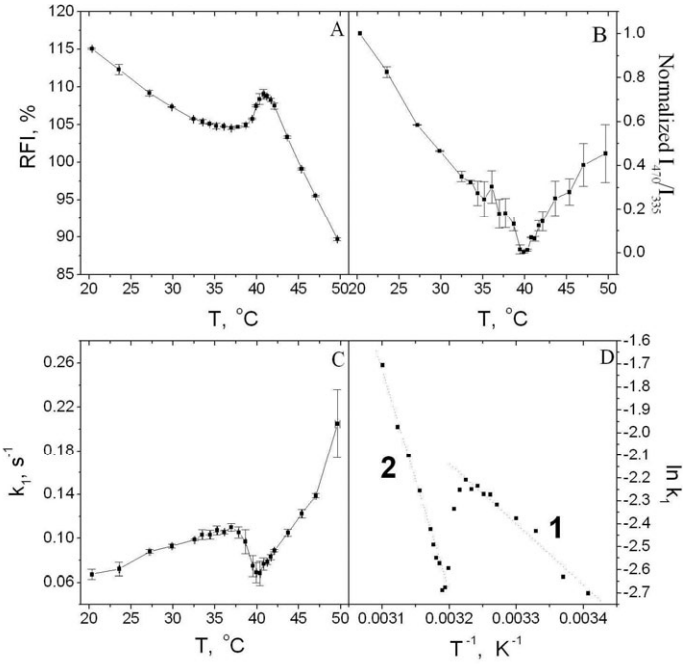

Fig. IV.3.1. The dependency of relative fluorescence intensity (RFI) *(panel A*), normalized ratio of emission intensities when excited at 470 or 335 nm (*panel B*), rate coefficient of reduction (*panel C*) for DPPN. In *panel D* the Arrhenius plot of rate coefficient below (1) and above (2) T_m are shown.

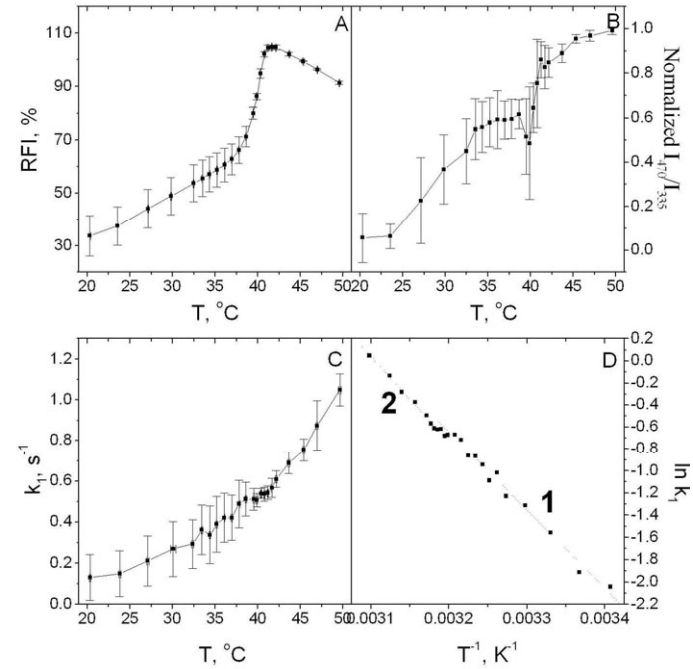

Fig. IV.3.2. The dependency of relative fluorescence intensity (RFI) *(panel A*), normalized ratio of emission intensities when excited at 470 or 335 nm (*panel B*), rate coefficient of reduction (*panel C*) for NBD-PC. In *panel D* the Arrhenius plot of rate coefficient below (1) and above (2) T_m are shown.

An additional interesting feature is that the rate coefficient for the reduction of the NBD moiety of DPPN is observed to have a minimum at T_m (*panel C*). However, to understand the features of the temperature dependency better, let us first take a look at the Arrhenius plots below and above T_m (*panel D* in Figs. IV.3.1 and IV.3.2). It can be seen that for NBD-PC the rate of reduction shows simple Arrhenius-like kinetics and is not significantly altered by the phase change, likely suggesting that most of the fast reduced NBD of NBD-PC is similarly available on the surface of both NBD-PC enriched in domains and dispersed in fluid phase. However, for DPPN we can see a steeper temperature dependency above the phase transition temperature and a weaker temperature dependency below it, corresponding to the apparent activation energies of 80 and 22 kJ/mol (or 31 and 8.4 kT/molecule at T_m), respectively. The higher apparent activation energy in the fluid phase implies that the probe is less available for reduction. Moreover, when approaching T_m from the fluid phase the rate of reduction decreases with a slightly stronger rate than expected based on a linear fit. Accordingly, at T_m there appears to be a genuine minimum (instead of just a stepwise change from gel to fluid phase temperature dependency). This agrees with the I_{470}/I_{335} data for DPPN, though inherent temperature dependent changes may complicate the interpretation of intensity ratio data. A simple explanation would be that at T_m the DPPN headgroup adopts a conformation in which the NBD moiety is immersed deeper inside the bilayer. A similar phenomenon for PC headgroups has been suggested based on extensive ζ-potential measurements (Makino et al., 1991).

4. NBD populations revealed by excitation band absorptivity difference (OP II)

Anisotropy measurements were also made using both absorption bands at 470 (r_{470}) and 335 nm (r_{335}) for excitation. Two things should be noted. First, there is no *a priori* reason that the anisotropies of the probe excited at two different wavelengths should be the same, even if the probe is in exactly the same environment. Second, anisotropy is affected equally by the changes in probe mobility and fluorescence lifetime. For the first note the reason is that at different excitation wavelengths the angle between the excitation and the emission (transition) dipole moment may be different, leading to a different fundamental anisotropy r_0. The dependence of r_0 on this angle β may be expressed by (Lakowicz, 1999):

$$r_0 = \frac{2}{5}\left(\frac{3\cos^2\beta - 1}{2}\right).$$

The different decay processes in turn begin to decrease the value of anisotropy from this value of fundamental anisotropy. For the single exponential decay with simple, isotropic rotation rates the steady-state anisotropy would simply be (Lakowicz, 1999):

$$r = \frac{r_0}{1 + (\tau/\theta)},$$

where τ is the fluorescence lifetime and θ is the rotational correlation time. Now, taking a look at Fig. III.4.1 we may note that in the case of DPPN the r_{335} has values slightly smaller than r_{470} at all temperatures, and the same applies for NBD-PC above T_m. This small difference in anisotropy most likely originates from different fundamental anisotropies. The value of β for the 470 nm band is 25° (Thompson et al., 1984). If we for the sake of argument apply the simplest decay model (equation shown above) to roughly estimate the required changes, we can note that the difference of 1.00°±0.65° in β would be sufficient the explain the slightly lower values of r_{335}.

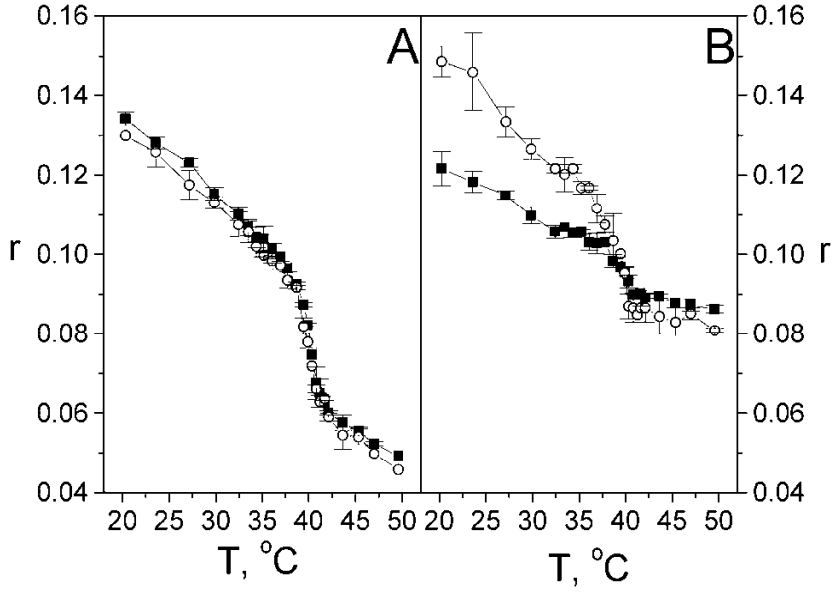

Figure III.4.1. The anisotropies of DPPN (*panel A*) and NBD-PC (*panel B*) at excitation wavelengths 470 nm (black squares) and 335 nm (white circles).

As discussed above, the response of band absorptivities to polarity is different, and this affects the absorptivities in different environments. Accordingly, in a less polar environment the 335 nm band absorbs more relative to the 470 nm band. The result is that if there are two populations of probes, of which one population is in a less polar surroundings and the other in a more polar surroundings, we would expect that a larger fraction of fluorescence comes from the less polar surroundings when excitation is at 335 nm. If there are additional differences e.g. in probe mobility or lifetime in these environments, this would induce a difference in anisotropies measured using these two wavelengths. This kind of behaviour is seen for NBD-PC below T_m. More specifically, the values of r_{335} are higher than those of r_{470}. This implies that 1) there indeed are two populations, 2) either the population in an apparently less polar environment is less mobile or its fluorescence lifetime is shorter. Of the two alternative scenarios the former appears more likely at the first look, as the less polar environment typically results in longer lifetimes for NBD (see e.g. Lin and Struve, 1991; Chattopadhyay et al., 2002).

However, the decrease in anisotropy at the main transition could largely result from decreased self-quenching, as suggested by the increased intensity. It is thus possible that the higher anisotropy derives from shorter lifetimes, if the environment is less polar for the segregated NBD-PC and more polar for NBD-PC dispersed in the DPPC matrix.

If we compare the behaviour of the difference r_{470}-r_{335} for NBD-PC to DSC scans, we may observe that $d[r_{470}-r_{335}]/dT$ reproduces the DSC peaks (Fig. III.4.2). There are two peaks in the endotherm, the first one corresponding to probe-enriched phase. Not surprisingly, in the curve based on fluorescence data the contribution of the probe-enriched phase appears to be overrepresented.

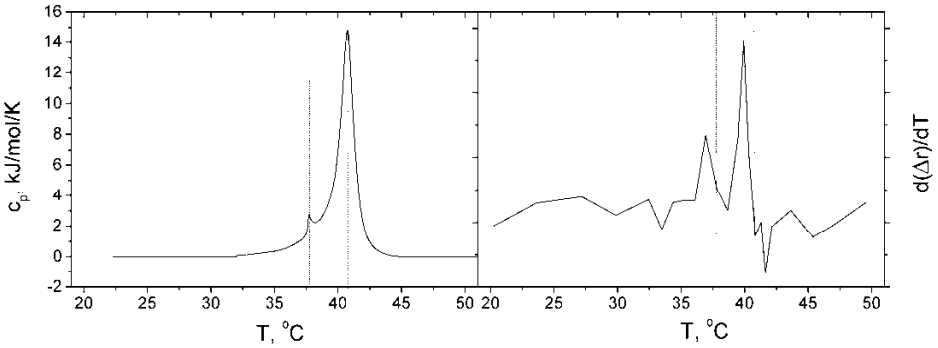

Figure III.4.2. Panel on the left shows the DSC scan of the DPPC:NBD-PC=97:3 LUVs. Panel on the right displays numerical derivative of the difference r_{470}-r_{335} (see Fig. III.4.1).

5. Coupling of the lipid/water free area to bulk surface tension (OP III)

While studying kosmotropes we observed that the surface tension of aqueous kosmotrope solutions for different kosmotropes did not show a direct correlation to the bulk osmotic activity. In Fig. III.5.1 the surface tension change $\Delta\gamma$ (compared to pure water) vs. the

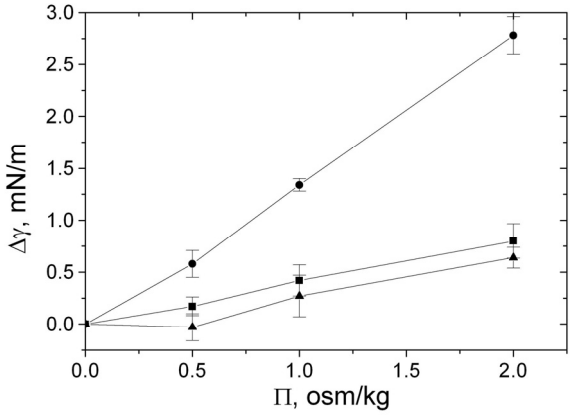

Figure III.5.1. The air/water surface tension change caused by betaine (squares), sucrose (circles) and choline (up triangle) at different osmotic pressures.

bulk osmotic activity is shown for sucrose, betaine, and choline. When the osmolarity is equal for all the kosmotrope solutions, sucrose has the strongest effect on surface tension, betaine comes second, and choline has weakest effect, though the difference between betaine and choline could derive from experimental error. It was of interest to study whether the effects on lipid bilayers would correlate better with osmolarity or with $\Delta\gamma$. More importantly, however, the data shows that the interactions with interfacial water are of great importance to the observed surface tension effects, and cannot be explained by the different osmotic activity of the solutes (see section I.3.2).

5.1. Bulk surface tension increment results in lipid area decrement

Laurdan GP was used to report on the changes in lipid molecular area in different solutions. This was based on the correlation found by Bagatolli et al. (1998) who described that for similar groups of lipids (such as groups of zwitterionic lipids, glycolipids, and anionic lipids) the GP value has a linear correlation to the intermolecular

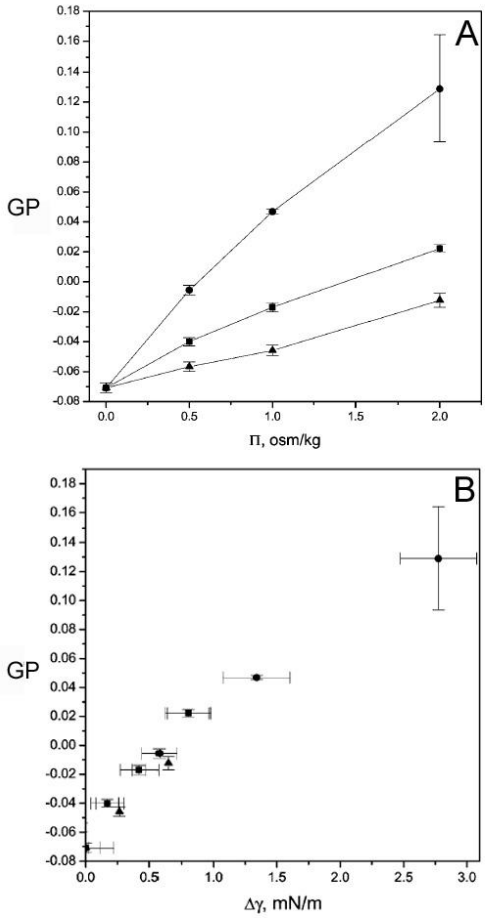

Figure III.5.2. The dependence of Laurdan GP value on osmotic activity Π (as osm/kg) (*panel A*) and air/water surface tension change $\Delta\gamma$ (*panel B*). In both panels circles indicate sucrose solutions, squares betaine solutions and triangles choline solutions. When comparing panels A and B it is obvious that the effects of different kosmotropes fall more or less on a single curve when data is plotted against $\Delta\gamma$ instead of Π.

distance at the level at which Laurdan resides, i.e. at the level of the carbonyl groups. This applies also for lipids below and above T_m. We will use the term interbackbone distance to describe this distance. Combining this distance with the backbone radius one may evaluate the area per lipid molecule.

GP vs. osmotic activity is shown in *panel A* of Fig. III.5.2, and GP vs. $\Delta\gamma$ is shown in *panel B* of Fig. III.5.2. The molecular areas based on the GP values and the relation in Bagatolli et al. (1998) are plotted against $\Delta\gamma$ in Fig. III.5.3. It can be easily seen that for these kosmotropic solutes lipid binding is not needed as an explanation for their different effects at similar osmotic activities. Instead (almost) all the deviation is contained in their effects on the surface tension of the air/water interface, suggesting that the energetics are related to the same structural factors that contribute to the kosmotropic effects of these compounds and that make the partition into the air/water interface unfavourable.

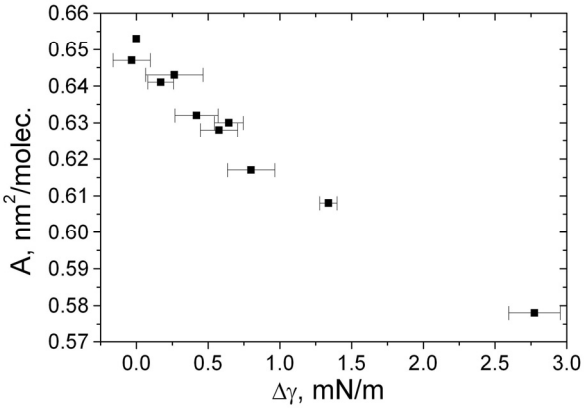

Figure III.5.3. The lipid molecular area calculated from Laurdan GP vs. surface tension change at air/water interface.

5.2. On lipid-water interfacial tension

As discussed in section 4 of the introduction, there is no net tension or net pressure for bilayers in equilibrium. Otherwise the bilayers would either contract or expand. However, they do have tension and pressure components which cancel each other out. Furthermore, these components act at different depths in the bilayers. The interfacial tension is mostly present at the lipid/water interface while the balancing pressures are mostly located deeper inside the bilayer. As monolayer techniques are versatile for many purposes, there has been a considerable effort to evaluate the monolayer interfacial tension that is equivalent to bilayers. Various experimental methods suggest that this apparent interfacial tension in bilayers is approx. 30–40 mN/m (see Marsh, 1996 for review).

Utilizing the above data we attempted to undertake a new approach to evaluate this pressure. We made the simplest assumption possible and assumed the changes in interfacial tension are additive, meaning that $\Delta\gamma$ is equal for air/water and lipid/water interfaces, i.e. $\Delta\gamma_{lipid/water}=\Delta\gamma_{air/water}$. The second simplifying assumption was that the observed area decrease (at least nearly) completely balances the increase in free energy due to the increase in the lipid/water interfacial tension. Now, for the initial interfacial

tension $\gamma_{lipid/water}$ and its change we get the relation $A\Delta\gamma_{lipid/water}+\gamma_{lipid/water}\Delta A=0$. Rearranging yields

$$\Delta\gamma_{lipid/water} = -\gamma_{lipid/water} \times \frac{\Delta A}{A}$$

Next, by varying the initial $\gamma_{lipid/water}$ we minimize the sum

$$\sum_i \left(\frac{\Delta\gamma_{lipid/water,i} - \Delta\gamma_{air/water,i}}{\sigma_{air/water,i}} \right)^2 = \sum_i \left(\frac{-\gamma_{lipid/water} \times \frac{\Delta A_i}{A} - \Delta\gamma_{air/water,i}}{\sigma_{air/water,i}} \right)^2$$

where i represents the identification for the solution, and $\sigma_{air/water}$ is the error in the measured $\Delta\gamma_{air/water}$. Due to the large deviation in GP values (and therefore ΔA values) for the 2 osm/kg sucrose solution, which are most likely related to technical problems in the measurement, it was left out from the minimization procedure. The minimum for the weighed sum of squares is achieved with the value $\gamma_{lipid/water}$=18.5 mN/m. As this should equal the real value of the lateral pressure, known to be in the range of 30–40 mN/m (Marsh, 1996), the value is obviously wrong. (In the original publication the good agreement obtained by calculating the lateral pressure in a manner similar to monolayers lead us to make an error regarding this point.) The error cannot be accounted for by involving more complex models such as that of Rawicz et al. (2000), as much larger changes in lipid–water interfacial tension would be needed to explain the area changes. Basically, two possibilities exist. First, with a change in hydration the behaviour of the phospholipid membrane lateral pressure vs. area is shifted towards smaller pressures at a smaller area. Second, it may be that the interfacial free area reported by Laurdan fluorescence is to some extent affected by occupancy of the various osmolytes in the lipid/water interface. In this case, the occupancy must directly reflect the difference in interactions in bulk and interfacial water for each solute. However, the latter alternative suggests that the interactions with interfacial water should be more likely for those with the strongest effect on γ, which appears unlikely. A decrease in the amount of interfacial water at the level of the lipid headgroups together with a change in the properties of bulk water thus appear as possible candidates to contribute to the changes in membrane area. Yet, another attractive alternative is that changes in the properties of water cause the Laurdan signal to deviate from the GP vs. interbackbone distance linearity otherwise observed for zwitterionic phospholipids. In this case the area changes are overestimated due to a change in the properties of interfacial water. Nevertheless, the response accurately reflects a response of clean water surfaces.

6. Membrane interactions of the anaesthetic pregnanolone (OP IV)

The results suggest that pregnanolone interacts with membranes by dipole potential and by hydrophobic interactions which are dependent on the headgroup spacing and the phase behaviour.

6.1. Binding of pregnanolone to monolayers

As anesthetics generally appear to decrease the dipole potential (Qin et al., 1995; Cafiso, 1998), it was of interest to test the binding of pregnanolone to monolayers whose dipole potential was varied with dipole potential decreasing phloretin or dipole potential increasing 6-ketocholestanol. Additionally, cholesterol was included due to its effect of increasing the headgroup spacing, which has been considered especially in terms of the umbrella model (Huang and Feigenson, 1999). The findings show that binding of pregnanolone increases in case of monolayers that contain either cholesterol or 6-ketocholestanol whereas binding drastically decreases for monolayers containing

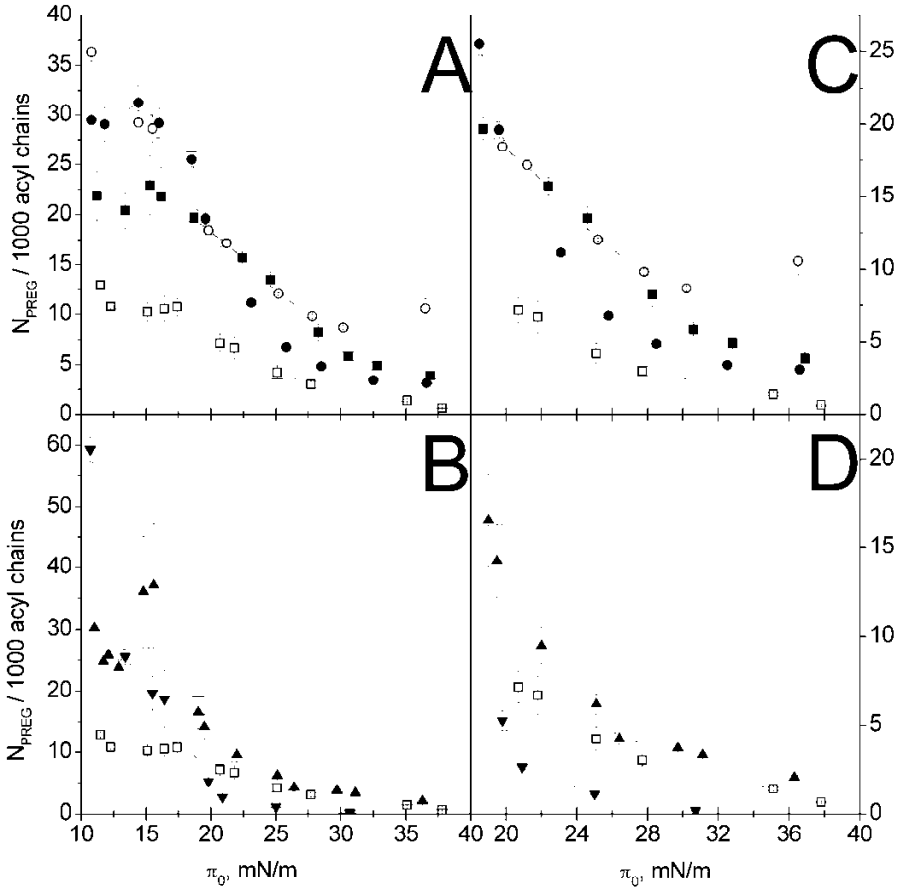

Figure III.6.1. The effect of monolayer composition on pregnanolone association. In *panel A* the effect of increasing the headgroup spacing by POPC and cholesterol is shown. POPC (○), DPPC (□), DPPC:cholesterol, 80:20 (■), and 60:40 (●) (mol:mol). In *panel B* the effects of dipole potential modulators are illustrated. The series are DPPC:phloretin, 80:20 (▼), DPPC (□), and DPPC:6-ketocholestanol, 80:20 (▲). The high-π data region of panels A and B which is devoid of phase transitions and more closely resembles the bilayer state is shown in *panels C* and *D*, respectively

phloretin (Fig. III.6.1). Further, the effect of cholesterol is much stronger than that of 6-ketocholestanol, although 6-KC increases Ψ more strongly, suggesting that some additional feature is mainly responsible for the binding-enhancing effect of cholesterol.

6.2. Pregnanolone effects on dipole potential

Motivated by the knowledge on the effect of other anaesthetics on Ψ, and by the effect of dipole potential modifiers on the binding, we set out to test the effect of pregnanolone on the dipole potential of phosphatidylcholine and phosphatidylcholine:cholesterol bilayers using di-8-ANEPPS as an indicator dye. The results revealed pregnanolone to decrease the dipole potential as expected (Fig. III.6.2). This provides an easy framework to also explain the effect of dipole potential modifiers on binding: increasing the dipole density of the membrane makes insertion of an oppositely oriented dipole more favourable, while decreasing the dipole density works in the opposite direction. As a result, the system again follows the familiar Le Chatelier's rule.

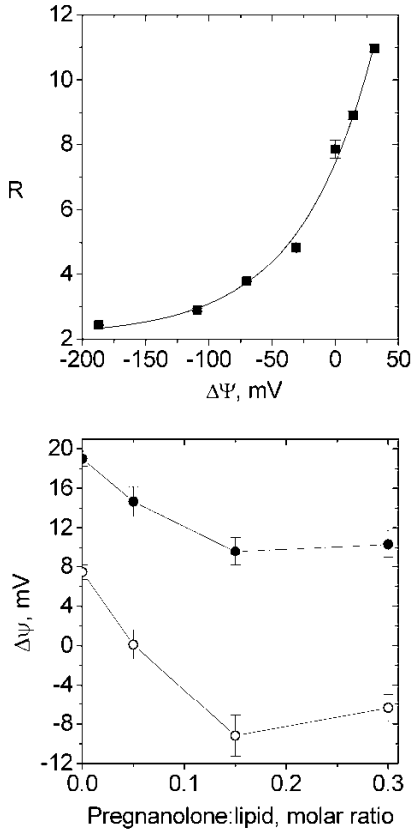

Figure III.6.2.
Upper panel shows the fluorescence intensity ratio of di-8-ANEPPS as a function of ΔΨ, measured for DPPC:6-ketocholestanol (90:10 and 95:5, mol:mol) and DPPC:phloretin (95:5, 90:10, 85:15, and 75:25) LUVs with the corresponding ΔΨ values of +31, +14, -31, -70, -109, and -187 mV (D. Cafiso, *personal communication*). A first-order exponential fit was used with χ^2=0.12953. Data points represent the average of three measurements and error bars show S.D.
Lower panel shows the effects of pregnanolone on the membrane dipole potential in DPPC (○) and DPPC:cholesterol, 90:10 (●) LUVs assessed by di-8-ANEPPS fluorescence. Final lipid concentration was 400 μM in 5 mM Hepes, 0.1 mM EDTA, pH 7.4. Temperature was 45 ^{o}C. Data points represent the average of three measurements and error bars show S.D.

6.3. Prodan reports changes in interfacial dynamics

We could observe pregnanolone to have significant effects on the GP of Prodan in bilayers. More specifically, pregnanolone increased the GP value in both the gel and fluid phase matrices (see Fig. III.6.3). The increase in GP implies that Prodan on average is in a less hydrated or in a less hydrogen-bonded state, thus apparently the surroundings are more rigid or more crowded by other molecules than water.

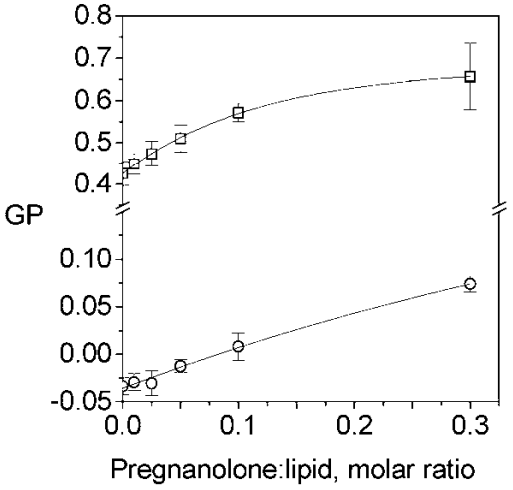

Figure III.6.3.
The effect of increasing pregnanolone:lipid ratio on the GP of Prodan in DPPC liposomes at 30 (□) and 50 °C (○) fitted with a single exponential decay as a guide for the eye. The data points represent averages of three measurements, and error bars represent standard deviation. The lipid concentration was 50 µM in 5 mM Hepes, 0.1 mM EDTA, pH 7.4.

6.4. Small effects on acyl chain order

In our experiments we noticed that pregnanolone had a negligible effect on the acyl chain order as reported by DPH anisotropy (Fig. III.6.4). While due to a difference in the lipid concentrations the DPH anisotropy values cannot be directly compared with the dipole potential results, the Prodan measurements were done at the same lipid concentration as the DPH anisotropy measurements. The considerable effect on Prodan GP and the negligible effect on DPH anisotropy thus suggest that pregnanolone affects the lipid/water interface more than the bilayer core. Considering the Prodan results in this light, it appears likely that interfacial crowding by pregnanolone is more important than any rigidifying effect. Thus, the increased headgroup spacing due to the presence of cholesterol could mechanistically lead to increased binding.

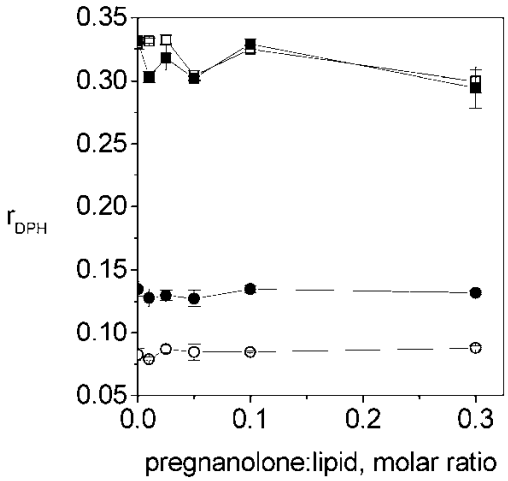

Figure III.6.4. Fluorescence anisotropy of DPH as a function of pregnanolone content. The effect of pregnanolone on gel (□) and fluid phase DPPC (○) LUVs as well as gel (■) and fluid phase DPPC:cholesterol (90:10, ●) LUVs was measured. Temperatures were 30 °C for gel phase and 45 °C for fluid liposomes. Final lipid concentration was 50 μM in 5 mM Hepes, 0.1 mM EDTA, pH 7.4.

PART V: DISCUSSION

1. Main phase transition

1.1. NBD derivatives probing the DPPC main transition

The fluorescence intensity of NBD-labelled probes in the DPPC matrix is mostly governed by self-quenching, especially for NBD-PC which demonstrates pronounced self-quenching in the gel state. Nevertheless, by assessing several features of the same system, we could obtain a better perspective of certain aspects of phase behaviour. A particular feature evident for NBD-PC is that there appears to be two populations below T_m based on the changes in r_{335} and r_{470} (*panel B* of Fig. III.4.1). However, the nature of the two populations is more difficult to explain. As $r_{335}>r_{470}$ either should the fluorescence lifetime of the population with the higher ε(335 nm)/ε(470 nm) ratio be lower or the mobility of this population restricted. Whichever is true, the populations are likely related to the extensive segregation of NBD-PC when present at the large mole fractions used here. Three possibilities are evident:

1) The high ε(335 nm)/ε(470 nm) population represents NBD-PC dispersed outside the segregated domains.

2) In the NBD-PC-enriched domains the NBD-PC molecules are connected by inter-NBD hydrogen bonds forming aggregates similar to those observed in monolayers (Tsukanova et al., 2002). This would require, however, that in these aggregates the

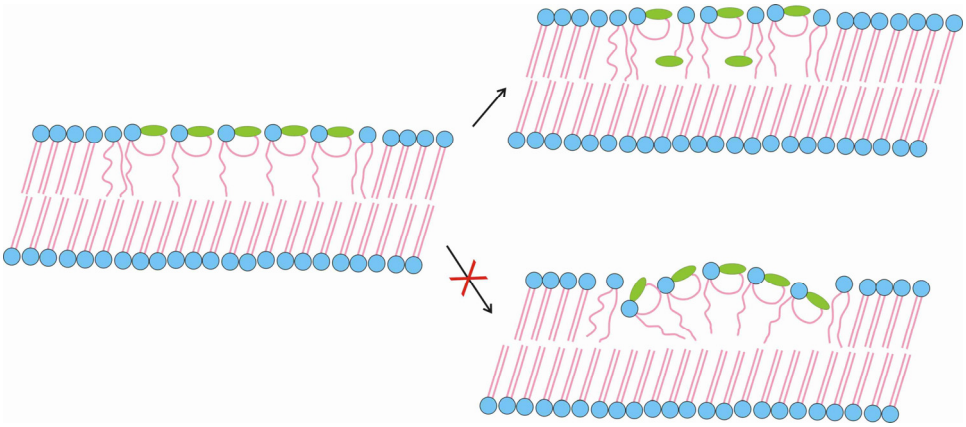

Figure V.2.1. A model to explain the two populations of NBD-PC in a gel phase matrix. Left, phase separation of NBD-PC in the gel phase is shown. NBD-dodecyl attached to the *sn*-2 position of glycerol backbone is known to be looped so as to bring the NBD into the interface. Thus effectively NBD-PC has only one acyl chain and a very large headgroup, suggesting that there is considerable curvature stress within NBD-PC enriched domains. This stress could be relieved by curving the interface (lower bilayer on right), but this would increase the area of the interface considerably, creating curvature stress elsewhere or inserting many highly polar phosphocholine moieties into bilayer core. Accordingly, this is unlikely to happen. Another way to relieve the curvature stress is to insert some of the moderately polar NBD-dodecyl moieties deeper into bilayer (upper bilayer on right).

ε(335 nm)/ε(470 nm) ratio is high, although they are in a polar environment.
3) When the X_{NBD-PC} is high, the domains enriched in NBD-PC also have a very high X_{NBD-PC}. These domains are likely to display significant curvature stress, due to the effectively very large headgroup of NBD-PC (PC and one looped-back NBD) with only one acyl chain remaining deeper in the hydrophobic part. This curvature stress could be relieved by partitioning of some of the NBD deeper into the hydrophobic part, where a higher ε(335 nm)/ε(470 nm) is expected, and a more restricted mobility is also possible. Yet for these NBD moieties the higher anisotropy for this population, apparently in less polar surroundings, would likely largely originate from the decreased lifetime (due to high self-quenching) in these NBD-lipid enriched domains.

At present the data does not allow us to make a clear distinction between the possibilities. However, the prediction for all alternatives is that this behaviour should diminish and possibly disappear for a sufficiently small X_{NBD-PC}. For the first alternative, we would expect the values of r_{470} to increase to the level of the r_{335} values. For the other two alternatives the r_{470} would correspond to the anisotropy of dispersed NBD-PC, and with decreasing X_{NBD-PC} we would expect the value of r_{335} to decrease to the same level as r_{470} values.

More interestingly, some of the behaviour of the DPPC matrix itself is perhaps revealed by the measurements with DPPN. The deepest location of the headgroups at T_m has been suggested based on ζ-potential measurements (Makino et al., 1991). The minimum in the reduction rate as well as in ε(470 nm)/ε(335 nm) are certainly in accordance with this suggestion. Langner et al. (2000) reached a similar conclusion based on a fluorescence spectroscopic study of fluorescein-labelled DPPE, i.e. DPPF, suggesting that the effects are not probe specific but reflect the behaviour of the matrix lipid, or at least reflect the general form of interactions with trace impurities. Accordingly, it would appear that interactions at the headgroup level and/or with water change in a complex way during the phase transition.

The importance of interactions with water is underlined by a recent molecular dynamics simulation of the phophatidylcholine pretransition (Vries, de, et al., 2005). The periodicities of ripples and the thickness of different ripple sections have been well characterized (see Nagle and Tristram-Nagle, 2000), yet the exact molecular level structure of the ripple phase has remained elusive prior to the MD simulation which reproduces all the structural parameters of the ripple phase. The results of the simulation show that the thinner segments of the ripple phase are interdigitated (see Fig. I.1.3), while the thicker segments are in a gel-like state.

1.2. On the role of the lipid/water interface in phospholipid main phase transition

Not only is the headgroup hydration changing at the transition, as discussed above, but as the spacing of phospholipids and the level of exposure of their hydrophobic parts are changing, the interfacial tension is changing, too. It is obvious that this makes an important contribution to the energetics of the main transition. Obviously then, because solutes have different effects on surface tension, these effects should be taken into account when considering solute effects on T_m and $\Delta_m H$.

More interesting changes take place. Polar headgroup and interfacial water organization have been found by fluorescence (Jutila and Kinnunen, 1997, Söderlund et

al., 1999; Metso et al., 2003), IR (Mellier and Diaf, 1988; Mellier et al., 1993), and dielectric spectroscopy (Enders and Nimitz, 1984) to show discontinuities slightly (up to a few degrees) below T_m. Most remarkably, the DPH fluorescence lifetime distribution collapses at 4 to 2 degrees below T_m, indicating the penetration of water into the bilayer (Gratton and Parasassi, 1995). The importance of headgroup hydration is further underlined by the new MD simulation study showing that $P_{\beta'}$ phase is in fact a phase formed by interdigitated and ordinary gel phase segments. A key factor appeared to be that some of the phospholipids in this cooling jump type of simulation retained their L_α phase hydration shell despite their ordered chains, resulting in an effectively large headgroup and driving the system to form interdigitated segments (de Vries et al., 2005). Obviously, when a sample is heated, at some the point hydration shell of the phospholipids changes resulting in the interdigitated segments of the $P_{\beta'}$ phase. Pretransition is nevertheless connected to the main transition and obviously the acyl chain order must play some role. A feasible explanation for these initial events can be obtained from the model of Heimburg (2000) (see section I.1.3). The formation of line defects takes place as a result of an increase in acyl chain disorder of some lipids, but in fact not all of the lipids in line defects have fluid like chains, instead their hydration obtains a fluid like nature, to balance the defect. Because the volume of lipids does not increase, the lipids interdigitate. Some of the anomalous phenomena just below T_m may be related to the reorganization from interdigitation in the system, as the fraction of the ripple phase decreases during transition. This might be related to the observed anomalies. As a note we may observe that the new view of the $P_{\beta'}$ phase structure gives a more uniform view about the effects of alcohols on the phospholipid phase behaviour. At low concentrations various alcohols stabilize the $P_{\beta'}$ phase, decreasing T_p much more than T_m. At somewhat higher concentrations the interdigitated $L_{\beta I}$ phase coexists with the $L_{\beta'}$ and $P_{\beta'}$ phases, while at even higher alcohol concentrations both $L_{\beta'}$ and $P_{\beta'}$ phases are replaced by the interdigitated $L_{\beta I}$ phase (Loebbeke and Cevc, 1995). These observations make a logical connection with the headgroup spacing increasing effect of alcohols.

However, the observation of the interfacial headgroup reorientation by us and others (Langner et al., 2000; Makino et al., 1991) is perhaps more likely to be related to an increase in the length of the phase boundary, possibly representing the orientation of boundary lipids. For more discussion on the possible role of phase boundaries, see Alakoskela and Kinnunen (2004).

2. Dipole potential

2.1. Interactions of ions with neutral bilayers

Most commonly, ion interactions with charged lipids are studied, and a plethora of publications about various aspects can be found. However, ions also associate to neutral bilayers (e.g. Makino et al., 1991). The associating ions give bilayers a net charge, and these ions also affect the dipole potential in a way that follows the Hofmeister series (Clarke and Lüpfert, 1999). However, as our studies suggest, an association may not necessarily be needed but the differences may be mediated by the effects on interfacial tension. Thus care should be taken in interpretating the effects of ions as binding, especially since changes in the lipid molecular area change the dipole potential (Clarke,

1997), and since the effects of ions on surface tension follow the Hofmeister series, which to large extent results from the solvation energy of ions and the dispersion interactions (see Boström et al., 2005, and references therein). Nevertheless, our results with the anion dithionite and the NBD-labelled lipid derivatives in different matrices clearly demonstrate that dipole potential in turn has a significant effect on the kinetics of ion penetration into the lipid/water interface. Therefore direct effects of dipole potential on ion binding are most likely, too, in accordance with the study assessing the effect of ions on dipole potential (Clarke and Lüpfert, 1999). An effect directly linked to dipole potential is expected, as DHPC has a similar hydration pressure compared to the DPPC, but as it nevertheless yielded results that agree with expected dipole potential change. This makes an effect related to the hydration unlikely unless hydration pressures are only loosely connected to water ordering at the surfaces, which appears as a possibility. A change related to the headgroup orientation of DPPN appears unlikely, as NBD attached to the tails and the headgroups produced similar results. However, a change in headgroup orientation of the bulk lipid is expected to take place, and this may contribute. Anions decrease Ψ, presumably by binding to sites in the inner positive region of the dipole potential generating layer (Clarke and Lüpfert, 1999). Increasing Ψ should thus promote anion binding, in keeping with the coincidence of maxima in Ψ and minima in zeta potential in binary phospholipid membranes (Luzardo et al., 1998). Thus, the decrease in reduction rate with increasing dipole potential implies that kinetic and not equilibrium partitioning effects are responsible for the observed effect, suggesting that the outer layer of negative partial charges creates a free energy barrier for the penetration of anions to the level of the positive partial charge layer.

2.2. Dipolar interactions of small molecules with bilayers

Evidently, as we know that the membrane dipole potential exists, the binding of dipoles to membranes should be affected by the dipole potential. Nevertheless, the extent of this effect is questionable, partly due to the complex environment of the lipid headgroup region and partly due to the difficulty of assessing the relative contributions of hydrophobic, dispersion, and electrostatic interactions in this environment. Obviously, when the hydrophobic effect is considerably stronger than dipolar interactions, the dipole potential is expected to have little effect. On the other hand, if dipolar interactions are of the same order of magnitude, the effects should be clear. For ions the results have shown a significant effect (Clarke and Lüpfert, 1999), yet, for ions we would expect dominance of electrostatic effects, except at high (>0.1 M) concentrations when the electrostatic effects are effectively screened and solvation and dispersion forces dominate (Boström et al., 2005). Our results on the pregnanolone association to monolayers of different compositions suggest that a modification of the membrane dipole potential has significant effects on the association of this weak amphiphile. One can speculate that also for highly polarizable, nondipolar molecules or atoms the immense field caused by dipole potential could have important effects (of course, dipole potential is just a simplification of the real (partial) charge distributions, which are a physical reality). Accordingly, this further clarifies the fact that simple oil/water partition coefficients should not be considered to have too strong of a predictive value for the association of a compound to the lipid/water interfacial region. In fact, the literature suggests that any analytical or simulation approach that attempts to predict the association of compounds in general to the

lipid/water interface cannot neglect any of the interactions related to solvation/solvophobicity, dispersion forces, polarizability, and electrostatics (see discussion on DPH and Xe in section III.4.8). This is not to say that predictions based on simplified schemes such as molecular dynamics simulations should not be made, on the contrary, but the accuracy of predictions should be verified experimentally for each solvent condition and compound separately. Gratifyingly, these approaches, unlike experiments, allow to select forces which are included, and thus to test the relative importance of different forces.

2.3. On the role of dipole potential in anaesthesia

Dipole potential has been suggested to affect several processes, including anaesthesia (see chapter I.5). Mainly these suggestions have been based on detected correlations, such as the similar effect of several anaesthetics on the dipole potential (Qin et al., 1995). However, the problem with respect to these approaches is that correlation can result from several factors as discussed in chapter I.5. In this respect, as the compounds tested by Qin et al. belonged to the group of volatile anaesthetics, it was of interest to test a steroid anaesthetic, as steroid anaesthetics belong to a separate group of anaesthetics. This difference is perhaps most clearly illustrated by the reports that the effects of neurosteroids appear to be limited to phylum Chordata (Oliver et al., 1991), whereas volatile anaesthetics affect all cells, including bacteria (Mehta et al., 1974). The conclusion of Oliver et al. was that the $GABA_AR$ is likely required for the anaesthetic effect of steroids. Nevertheless, steroid anaesthetics share many features with other anaesthetics, e.g. they are weak amphiphiles, their membrane interactions have been shown to correlate with their potency, and they appear to affect all lipids and proteins, similarly to volatile anaesthetics, as discussed in section I.6.2. Our characterization of the interactions of pregnanolone with lipid bilayers revealed pregnanolone, a steroid anaesthetic, to share another feature with volatile anaesthetics: it decreases the magnitude of dipole potential. While correlations such as this and that of the Meyer-Overton rule do not mean that the mechanism would be directly related to the dipole potential decreasing ability or the solubility to lipid headgroup level, it would nevertheless ease one's mind if the mechanism would explain these very general correlations.

One of the possibilities is that anaesthesia in general is lipid-mediated. However, our new results obtained with enantiomers of anaesthetic steroids suggest that at least this is not the case for steroid anaesthetics, as the far more potent natural allopregnanolone has exactly the same effect as its less potent enantiomer *ent*-allopregnanolone on every tested property of lipid phases of different model and biological membranes (Alakoskela, Covey, and Kinnunen, manuscript in preparation). Now, if we exclude this possibility at least in the case of steroids, then we must look for alternative explanations.

One of the alternative explanations is that the binding site on an important anaesthesia-mediating protein has a dipole and an amphiphilicity that requires the positive end of the binding dipole to be at the more hydrophilic end (see Fig. I.5.2). At least three other possibilities exist, and for two of these we must note that for the $GABA_AR$ the anaesthesia-mediating amino acids appears to be located in the lipid/water interface. If the anaesthetic in the bound state is completely shielded from the membrane, the equilibrium constant for the binding of an anaesthetic to membrane has no effect on the equilibrium constant for the binding of the anesthetic to protein (although some

confusion exists on this simple point, see Suezaki and Shirahama, 2002). Only limitation is that the protein/lipid and lipid/water equilibrium constants for anaesthetic association cannot be independently selected, but are limited by the fact that their product must equal the protein/water binding constant. Nevertheless, kinetic effects may perhaps arise in special circumstances. In the initial phase also these, even if present, are expected to be small, as the equilibrium concentrations dominate. In fact, for any set of simple reaction kinetics it is difficult to find any kinetic effects either, based on my own tests of modelling the kinetics as a set of differential equations using Matlab. Yet, the complex kinetics of proteins coupled to the complex phase behaviour of the real system may lead to kinetic effects. In such a case, a higher local mole fraction of anaesthetic may have significance. In this respect, it is interesting that our results with pregnanolone suggest that anaesthetics will be present at higher mole fractions in regions of higher dipole potential and larger cholesterol content, as illustrated in Fig. V.2.1.

However, if the anaesthetic retains some interactions with lipids even when bound to a protein, an intermediate binding affinity to lipids will be required for optimal protein binding in the equilibrium state. Obviously, based on our results, dipole potential is one of the factors affecting the partition coefficient. In the case of 50 % of the lipid-anaesthetic interactions still present when the anaesthetic is bound to protein, and assuming 40 mM total phospholipid in the body, the optimal equilibrium molar

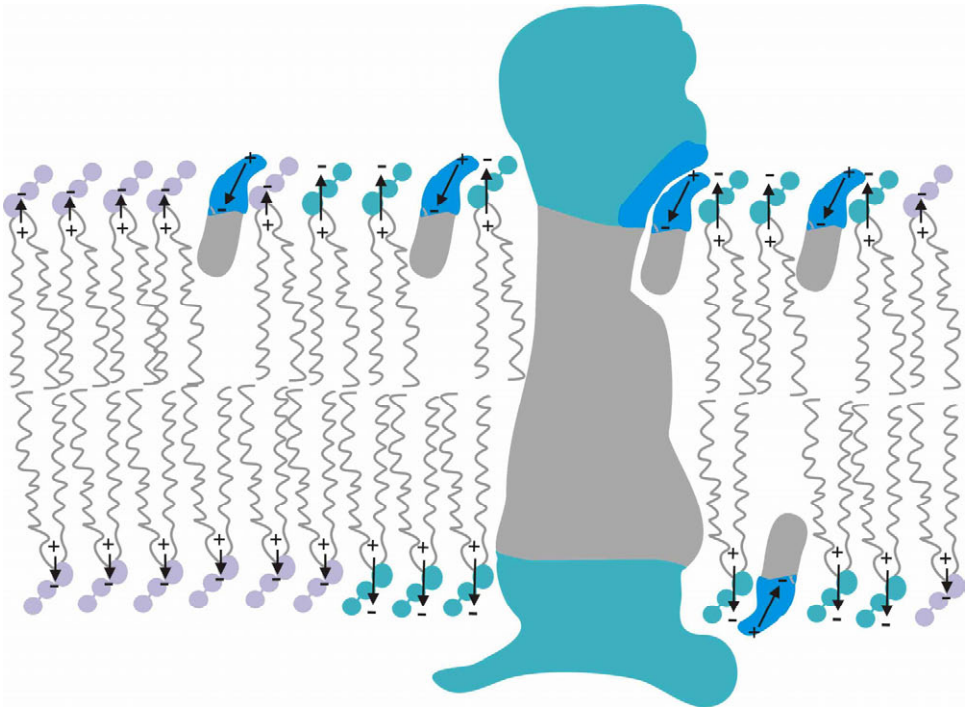

Figure V.2.1. Schematic illustration of anaesthetic distribution and binding in membranes. The lipid domain where the protein is located has a higher local concentration of the anesthetic (blue-grey molecules), as the lipids of this domain (green) have larger dipole potential. If the binding of the anaesthetic to the protein is shallow, the anaesthetic will retain some of its interactions with the lipid and the water surroundings, and these will affect the equilibrium constant for binding.

lipid/water partition constant would be approx. 1000, if the requirements by the protein binding site are in no way connected to the requirements of the lipid binding site. Clearly this value is far too small to explain the Meyer–Overton rule. However, shallow clefts in protein structure are likely to have polarity characteristics similar to those of the lipid headgroup region, as discussed frequently e.g. by Ueda and co-workers (see e.g. Ueda et al., 2002).

If anaesthesia is truly result of only direct binding to proteins, then the dipole potential decreasing effect of anaesthetics implies that clefts in proteins typically have a dipole organisation similar to that of the lipid/water interface. As membrane dipole potential results largely from water at the interface (Marrink, 1996), and as anaesthetics are known to affect lipid and protein hydration (Ueda and Yoshida, 1999), perhaps similar water dipole organization exists at the rims of weakly hydrophobic clefts at the protein surface.

3. Hydrophobicity, headgroup spacing, and interfacial tension

Hydrophobic interactions are nontrivial, and they are still a subject of active research and modelling. Similarly, while the thermodynamics of the solute effects on interfacial tension have been long known, advances in the detailed description of the origin of the energies have been only recently achieved (see e.g. Boström et al., 2001; 2005).

3.1. Hydrophobicity and headgroup spacing

For hydrophobic interactions there is an important distinction between the interactions of small and large particles with water. Typically, for small molecules the strength of the hydrophobic interactions increases with increasing temperature. However, important observable properties such as the solubility of the molecules in water show a different temperature dependence. The reason for this is that the thermal energy increases faster as a function of temperature than the strength of hydrophobic interactions, and solubilities or critical micellar/bilayer concentrations typically show a minimum close to room temperature (see Southall et al., 2002). Different behaviour is observed for macroscopic interfaces, e.g. a hydrocarbon/water interface, whose interfacial tension decreases continuously with increasing temperature and is devoid of the curious behaviour observed for individual hydrocarbon molecules in water (see Southall et al., 2002). Accordingly, behaviour is different for small and large interfaces, and intuitively one would expect that the size scale would be set by the order of magnitude of a few hydrogen-bonded water molecules. Indeed, the length-scale for a patch large enough to appear as a macroscopic surface for water is approx. 1 nm, at least in the case of protein surfaces (Huang and Chandler, 2000), whereas the typical centre-to-centre distance of liquid water molecules is approx. 0.28 nm (Head-Gordon and Hura, 2002). For intact lipid bilayers the hydrophobic interaction should thus mainly be of the kind as seen for large surfaces, i.e. simple behaviour characterized by a decrease in interfacial tension with increasing temperature, in order to agree with the length-scale estimate of Huang and Chandler (2000). Curiously, the effects of different osmolytes on lipid bilayers

correlate well with the effects seen at the air/water interface, suggesting that the interfacial exclusion of these compounds is the driving force in both situations.

Headgroup spacing is expected to affect both the interfacial tension, as this is dependent on the lipid area. (Note that I am using interfacial tension in this book in a non-standard manner, using interfacial tension to implicate the area derivative of interfacial free energy at a given area and temperature. Accordingly, it is not independent of the area as is true interfacial tension.) Additionally, an increase in headgroup spacing can be seen as introducing more hydrophobic patches on the membrane surface. In this sense, one would expect them to increase the binding of weakly interfacially active compounds to membranes, for instance anaesthetics. Indeed, in simulations cholesterol has been shown to give rise to interfacial cavities and to increase the association of small molecules to bilayers (Jedlovszky and Mezei, 2003). This agrees perfectly with our results for pregnanolone and cholesterol. The effect of cholesterol on headgroup spacing may be seen also in the light of the umbrella model (Huang and Feigenson, 1999). Briefly, this model suggests that due to the small headgroup of cholesterol the nearby phospholipid headgroups reorient to cover the hydrophobic patch created as an umbrella. This model suggests that the (maximum) solubility of cholesterol into bilayers is reached when the bilayers run out of the umbrella headgroups, and the model indeed gives good predictions for the maximum solubilities of cholesterol into different membranes. This simple view of cholesterol can thus explain both the solubility of cholesterol as well as the cholesterol effects on the association of other compounds, such as pregnanolone. A similar effect has been previously found for progesterone (Carlson et al., 1983). Also steroids have effects on the solubility of other steroids, e.g. progesterone increases lipid-solubility of estradiol (Heap et al., 1971).

3.2. Modulation of interfacial tension by small solutes

The thermodynamics of solute effects on equilibrium interfacial tension has been known ever since the introduction of the Gibbs adsorption isotherm. Basically, if the energy of a solute is higher near the interface or higher in the interface than in bulk, the surface concentration of the solute will exceed the bulk concentration and the surface tension will be lowered. Reciprocally, if the interfacial free energy for a solute is higher than in bulk, the solute will be depleted from a region near the interface. From now on, we will only concentrate on the case of surface tension increasing solutes. In equilibrium, the concentration of a solute will follow the Boltzmann distribution. A useful construction is the Gibbs dividing surface, which for surface-depleted solute divides the solution into the bulk with the bulk concentration, and into the interfacial region completely devoid of solute. Of course this is not what really takes place in the interface but is rather a tool of thought experiments. Now, we may think of the interfacial tension in terms of osmotic pressure and energy, as the situation is similar to any two compartments with different water activities (Parsegian et al., 2000). However, comparison with osmotic pressure does not take us far, as in the Gibbs dividing surface construction the depth of the dividing surface must either be fitted to surface tension data or assigned arbitrarily. To emphasize this point more, water in the surface is not organized in a similar manner as the bulk water, but a few layers of water molecules in the interface have less orientational freedom, for instance, and this may affect their interactions with solutes. For this reason it is possible that the coefficients for interfacial tension effects and for osmotic activity of a

solute may be different (Kunz et al., 2004). Another aspect worth mentioning is that the Gibbs dividing surface construction or the thermodynamic view in general makes no statements about the nature of the forces leading to the observed surface tension effects, and, accordingly, give no clue about the actual distribution of solutes near the interface. For example, monovalent salts have been known for over a hundred years to have ion-specific effects following the so-called Hofmeister series. These specific ion effects include the effects on surface tension. The quest of explaining these specific ion effects has only lately produced results once the solvation energies and polarizabilities (i.e. dispersion interactions) were accounted for (Boström et al., 2005, and references therein). Curiously, in some cases theory predicts two minima in the concentration of ions, suggesting that the concentration due to surface depletion does not necessarily just smoothly grow to the values of bulk concentration.

Importantly, although the measurement of surface tension increments due to solutes is notoriously error-prone due to surface active impurities, we do not expect our results to disagree much, as the effects on lipid bilayers agree very well with the measured surface tension effects. Thus, the disagreement between osmotic activity and surface tension effects in our results clearly imply that the interactions with the interfacial water layers or the different polarizability of the non-aqueous phase are important for the surface tension effects of biological osmolytes.

4. On the modulation of cell functions by membrane properties

The results of this study shed light on some of the effects of small solutes on bilayers and in turn on the effects that bilayer properties have on their interactions with these small solutes. Thanks to a large number of studies, which have been recently reviewed (Kruijff, 2004) it is now evident that the lipid surroundings of a membrane protein significantly affect the functions of the protein. Indeed, it appears that whenever an integral membrane protein can be successfully obtained as an active protein in large enough amounts and subsequently reconstituted, significant effects of the lipid surroundings on the activity of the protein can be demonstrated. Thus, the requirement of the right kind of a lipid environment appears at least at present to be a general feature of proteins, which is hardly surprising, since drastic changes in the physical properties of the environment are in question. Nevertheless, these modulatory effects by bilayers are usually much smaller than the activity changes induced by binding of specific protein ligands. It may be this very fact, together with the strict regulation of membrane properties by the adjustment of the lipid composition, that the effect of bilayer properties is often overlooked. Yet, one should observe that there is a conceptual difference in the effects of reaction cascades initiated by specific ligands and by effects that bilayer properties have on cellular function.

While it is difficult to quantitatively evaluate the relative effects of specific ligands and bilayer properties as long as we do not have complete understanding of the functions of a cell, the emerging models of a cell as a mathematically complex system of interconnected and interacting networks suggest that a simplistic view of observing the the activity of a single protein in the cell may not tell the whole truth of the relative importance of effectors. A large fraction, more than 30 % of the genes present in the human genome are expected to code integral membrane proteins, and a large fraction of

the remaining proteins are likely peripheral membrane proteins, suggesting that probably most proteins are membrane proteins (Mouritsen, 2005). Accordingly if we consider a single protein to present a single node, with connections of certain strengths to other nodes, we may visualize the effect of ligand binding as a drastic change in the strength of some connections from a single protein as well as subsequent downstream connections. As a result the cell may or may not shift into a new functional state. However, if we would use systems biology to understand the effect of the physical properties of bilayers, we should consider the relatively weak bilayer-mediated effects on most proteins by imagining a situation in which the strengths of most connections are changed by a small amount, that is increased or decreased depending on connection. Thus, one would surely expect that properties of bilayers could have a major impact on the behaviour of cells, in accordance with the strict regulation of bilayer properties.

Admittedly, even if we take for granted that bilayer properties can have a significant effects on cells, we are left with the question of whether changes in these properties are used by cells themselves for regulation, or are they simply regulated in order to maintain them at a constant value at all times. In the latter case, membrane properties would still be important when changed by foreign compounds or changing as a result of a malfunction in the regulatory system. Some of the properties related to the lipid/water interface appear to be more or less fixed by the growth temperature (Ginsberg et al., 1991; Jin et al., 1999). Yet, there is little data available with respect to the physiological regulation of the properties of the lipid/water interface. A recently published study of the dipole potentials of vertebrate plasma membranes found that the dipole potential of vertebrate brain plasma membranes varied between 293—306 mV, and the dipole potential of the kidney plasma membrane lipid extracts varied between 236—334 mV. Additionally, Na^+-K^+-ATPase activity was found to have an excellent positive correlation with dipole potential (Starke-Peterkovic et al., 2005). The large variation of the kidney lipid dipole potentials and small variations of the brain lipid dipole potentials could suggest that dipole potential has a role in regulation of protein activities between the cell types — or at very least, it raises the question why there is so very little variation between the dipole potentials of brain plasma membrane lipids. On the other hand, considering that cholesterol-rich domains formed by cholesterol and sphingomyelin have been suggested to have an important role in the plasma membrane organization (Thompson and Tillack, 1985; Simons and Ikonen, 1997; Epand, 2003), it is interesting to notice that equimolar mixtures of cholesterol and bovine brain sphingomyelin appear to have a dipole potential (412 mV) equal to that of pure egg-PC (415 mV), whereas the dipole potential of equimolar PC:CHOL mixtures have a considerably higher dipole potentials (493 mV) (McIntosh et al., 1992). (Please notice that the dipole potentials of the study by McInthosh et al. and that of Starke-Peterkovic et al. are not comparable, as the values of the former study were obtained from monolayers and those of the latter by dye-based method, using different calibration. See I.5.1. for more detailed discussion.) Considering that the preference for the sphingomyelin-cholesterol interaction appears to arise largely if not solely from the more saturated chains of natural sphingomyelins compared to natural phosphatidylcholines (see Holopainen et al., 2004, and references therein), it is tempting to speculate that perhaps sphingomyelin has been selected during evolution for its role in having saturated chains and participating in cholesterol-rich domains at least partly in order to have the same dipole potential as in the surrounding phase consisting largely of PC species. Yet, even if this were true, it would not necessarily imply that the primary reason would be to avoid disorders of protein function, but the equal dipole

densities of domains and the surrounding phase could also contribute to the stability of cholesterol-rich domains.

Another area of pure speculation lies in the selection of osmolytes by cells for the purposes of osmoprotection. It is known that e.g. *Listeria monocytogenes* employs a wide range of osmoprotectants in a hypertonic environment, including at least: betaine, carnitine, proline, proline-betaine, acetyl-carnitine, γ-butyrobetaine and 3-dimethyl-sulfoniopropionate (Sleator et al., 2003). These osmoprotectants are called compatible solutes as they tend to preserve protein structure. The mechanism by which compatible solutes stabilize proteins is by preferential exclusion that also leads to an increase in interfacial tension (Lin and Timasheff, 1996; Timasheff, 1998), although interfacial tension is not as useful a concept for proteins whose surfaces are uneven and heterogeneous, and interfacial energy would be more accurate. Interestingly, a mixture of urea and trimethylamine N-oxide is used by some organisms for the purpose of osmoregulation. Urea preferentially associates with proteins and denatures them, while trimethylamine N-oxide (TMAO) is preferentially excluded from the protein surface. The total effect of the mixture is to increase osmolarity but to avoid changes in the denaturation temperature (and thus likely avoid changes in protein dynamics) (Lin and Timasheff, 1994). Modelling has confirmed the experimental results that TMAO acts by enhancing hydrogen-bonding between water molecules and by being excluded from peptide surfaces (Bennion and Daggett, 2004). Considering these findings about osmoregulation and the different surface tension effects of osmolytes demonstrated by our measurements, it is tempting to speculate that maybe there are differences between the osmolytes employed by cells and that perhaps these differences are used by cells to adjust their response in different conditions.

At present we only have a few glimpses of membrane properties that are regulated, and even less is known about their modifications in response to changes in the environment (except perhaps in the case of temperature changes). More is known about the effects of bilayer properties on individual proteins, and on interactions of various compounds with bilayers. This study contributes to the latter category, and along with other similar studies suggests that cells must exert some control over the properties of the bilayer interface, if only to prevent any changes.

5. Conclusion

Understanding the lipid/water interface is important for understanding the interactions of the membrane with a wide variety of compounds. Interfacial free volume or hydrophobic patches, interfacial tension with associated water density depletion, orientational ordering of dipoles, and dipole potential all affect the interactions of phospholipid bilayers with ions, small compounds, and proteins. This work demonstrated some aspects of these phenomena.

Original publications I and II demonstrate that the interactions of lipid bilayers with soluble ions are sensitive to both the dipole potential and the lipid phase state. The effects of the properties of the interfacial region to binding of a small molecule are demonstrated in original publication IV. Original publication III demonstrated the lack of correspondence between osmotic pressure and surface tension as well as a good

correlation of surface tension effects and fluorescence of a probe in the interfacial region of lipid bilayers.

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