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Physical principles of membrane organization

J. N. ISRAELACHVILI,* S. MARČELJA AND R. G. HORN
*Department of Applied Mathematics, Research School of Physical Sciences (*Also Department of Neurobiology, Research School of Biological Sciences) Institute of Advanced Studies, Australian National University, Canberra A.C.T.* 2600, *Australia*

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I. INTRODUCTION

Membranes are the most common cellular structures in both plants and animals. They are now recognized as being involved in almost all aspects of cellular activity ranging from motility and food entrapment in simple unicellular organisms, to energy transduction, immuno-recognition, nerve conduction and biosynthesis in plants and higher organisms. This functional diversity is reflected in the wide variety of lipids and particularly of proteins that compose different membranes. An understanding of the physical principles that govern the molecular organization of membranes is essential for an understanding of their physiological roles since *structure* and *function* are much more inter-dependent in membranes than in, say, simple chemical reactions in solution. We must recognize, however, that the word 'understanding' means different things in different disciplines, and nowhere is this more apparent than in this multidisciplinary area where biology, chemistry and physics meet. To a biologist once a crucial experiment has succeeded in resolving an important issue the problem is deemed to have been solved, new concepts are introduced, and the issue is now 'understood'. Physicists, however, require a quantitative theoretical framework for their discussions, with the expectation that this also has some predictive capacity that can be tested experimentally, before some phenomenon may be said to be 'understood'. All the models of mem-

brane structure so far postulated fall into the biological rather than the physical definition of understanding: they are descriptive models rather than quantitative theories. In this article we review the experimental data relating to lipid and lipid-protein interactions with the aim of quantifying the physical and thermodynamic principles of membrane structure.

Langmuir was the first to appreciate the importance of considering the interactions between lipid molecules in determining the structures that they form. Langmuir, however, was more concerned with monolayers, and it was left to his contemporaries Gorter & Grendel (1925) to first demonstrate that the basic structure of biological membranes is probably a lipid bilayer. Subsequent models of membrane structure (Danielli & Davson, 1935; Davson & Danielli, 1952; Robertson, 1964) all retained the bilayer as an integral part of membranes, though other models were also proposed (Green & Perdue, 1966; Sjöstrand & Barajas, 1970). The present commonly accepted view of biological membranes is embodied in the fluid mosaic model (Singer, 1971; Singer & Nicolson, 1972) which envisages membranes as composed of proteins incorporated either wholly or partly in a fluid-like sea of bilayer lipids; these provide the structural framework of the membrane and allow the proteins to move about more or less freely in them. Further developments of the fluid mosaic model have been discussed by Capaldi (1974), Nicolson, Poste & Ji (1977) and Singer (1977). The model ignores lipid heterogeneity and tends to stress the planar bilayer and independence of proteins. But there are now experimental indications that the organization of lipids and proteins is more interdependent than was previously recognized. Indeed, it should be readily apparent that certain proteins – depending on their shape – cannot possibly fit into a lipid bilayer without distorting it from its original unstressed shape, i.e. some structural coupling is always necessary; this can have both structural and functional consequences as will be discussed.

Previous work on membrane structure did not address itself to the issue of intermolecular interactions and the thermodynamics of lipid and protein self-assembly. Thus the earlier theoretical work on micelles and monolayers was never developed to include bilayer and membrane forming lipids, and it is curious that none of the membrane models sought to explain why certain lipids form bilayers while others do not even though the lipids were often regarded as providing the structural framework of these assemblies. The lack of theoretical progress

concerning the thermodynamic basis of bilayer and membrane aggregation is, however, understandable since a rigorous application of statistical mechanics or the laws of thermodynamics to problems of self-assembly of multicomponent systems has always been difficult as was recognized by Onsager long ago (see Israelachvili & Ninham (1977) for a discursive review of this matter). On the conceptual side while the first law of thermodynamics definitely tells us whether some interaction is energetically favourable, the second law is much more vague since it only tells us of the *probability* of an event – and this only after we know all the other possibilities. The problem is particularly relevant to biological modelling of membranes and membrane-associated phenomena where some interaction (e.g. electrostatic, hydrogen-bonding, hydrophobic) may appear to be energetically favourable but is in fact highly unlikely on entropic grounds. Clearly some rigorous but preferably tractable thermodynamic principles are needed. The simple thermodynamic considerations of the fluid mosaic model are however too naïve; they provide necessary but insufficient criteria for a physical understanding of membrane structure if we also require an answer to the question 'why?' The interactions between membrane components and the way they pack together (i.e. favourably accommodate each other) must also be considered, as well as the thermodynamic laws of self-assembly, if we are to gain insight into the complex and highly specific organization of membranes at the molecular level.

The above comments do not imply that we should expect yet another model. On the contrary, the fluid mosaic model is probably the last of the celebrated membrane models. The structures of different membranes are now known to be so varied, complex, and even temperature-dependent, that a universal model cannot possibly hope to describe them all. The organization of some membranes can also be affected by protein binding to an underlying cytoskeleton of microtubules and microfilaments (Roberts & Hyams, 1979), or by the close approach of another membrane as occurs in myelin, thylakoids and cell junctions, and it may even change with time due to lipid and protein exchange with other membranes (Kader 1977). All this implies that membranes can no longer be considered as independent entities, which further complicates their modelling. It is perhaps for these reasons that reviews of the physical and structural properties of membranes have become more specialized, concentrating either on a particular membrane (Bretscher & Raff, 1975; Boggs & Moscarello, 1978*a*; Henderson, 1977)

or on specific aspects of membrane behaviour such as lipid-protein asymmetry (Rothman & Lenard, 1977; Lodish & Rothman, 1979), mobility and membrane fluidity (Shinitzky & Henkart, 1979), physical properties (Lee, 1975), gross membrane shape changes (Sheetz & Singer, 1974), phase transitions (Nagle, 1980), surface receptors (Nicolson, 1976), the role of lipids (Cullis & De Kruijff, 1979; Quinn & Williams, 1978), or cell surface properties (Poste & Nicolson, 1977).

There is another more important reason why it may actually be undesirable to expect further refinements in general membrane modelling, for membranes – unlike DNA, microtubules, viruses and proteins – are inherently fluid structures, and any 'model of membrane structure' accompanied as it usually is with pretty pictures can be very misleading. Instead, the recognition and proper application of the various intermolecular forces and thermodynamic laws that determine the complex dynamic organization of membrane components should be the primary concern in this area. This organization is governed by certain energetic, entropic and steric constraints which determine and set limits on the thermodynamically possible ways that the molecules can pack together. These principles apply to membrane assemblies just as they do to other macromolecular aggregates. Indeed, a unified theoretical approach to lipid and lipid-protein assemblies is essential if meaningful progress is to be made, and it is in this spirit that this review is written.

In the following sections we consider the experimental and theoretical evidence from a wide range of physical and biological studies. The emphasis will be on the general principles involved, and – where possible – on their quantitative application to specific situations. We have included only those aspects of membrane properties that we believe are explicable in terms of rigorous and testable thermodynamic principles. The reader will therefore find little discussion of the more speculative aspects of membrane modelling such as protein folding pathways in membranes, active transport, membrane bioenergetics and biosynthesis.

II. Structural properties of lipid bilayers

In spite of their great functional diversity, the basic structure of most biological membranes is a lipid bilayer. Lipid bilayers can be readily formed from both synthetic and biological lipids as vesicles, liposomes, multilayers or BLMs, and then studied under well-controlled conditions. Such studies (e.g. Papahadjopoulos, 1973) have provided a great deal of insight into many physical aspects of biological membranes, and their importance today remains undiminished.

Lipid bilayers comprise two physically very different environments. The *hydrophobic* bilayer interior is a relatively homogeneous region of hydrocarbon chains interacting mainly through fairly well-understood van der Waals attractive and steric repulsive forces. By contrast the *hydrophilic* head-group region containing hydrated and/or ionized (charged) groups in contact with the aqueous medium is far less understood. Here the interactions are very different: steric interactions, dipolar and electrostatic forces – involving specifically bound and free counterions, strongly-associated water and hydrogen bonds – all contribute in determining the physical state of the bilayer interfacial regions. In this section we shall examine in turn the physical states of the hydrocarbon and head-group regions of bilayers, and then go on to consider in this and the following sections how these two very different environments act together to give bilayers and membranes their highly versatile and complex properties.

A. *Interior of lipid bilayers*

Much information about the dynamic structure of the hydrocarbon chain interiors of lipid bilayers has been obtained from X-ray diffraction experiments. These experiments distinguish two fundamental physical states of order, further characterized by a number of finer differences and variations. At high temperatures, the X-ray pattern is characterized by a broad diffraction band at 4·6 Å, similar to that observed in liquid paraffins (Tardieu, Luzzati & Reman, 1973), indicating that the chains are in a fluid state. At lower temperatures, the chains are characterized by a sharp reflection at 4·2 Å, as well as some higher-order reflections. This corresponds to a state of stiff, fully-extended chains possessing rotational disorder and arranged in a two-dimensional hexagonal lattice. The higher-order reflections provide information about the finer details of these structures, e.g. the angle of tilt of the chains within the

Table 1

	Number of carbons per chain	T_t (°C)	ΔH_t (kcal/mole)
Dimyristoylphosphatidylcholine*	14	23·9	5·4
Dipalmitoylphosphatidylcholine*	16	41·4	8·7
Distearoylphosphatidylcholine*	18	54·9	10·6
Dimyristoylphosphatidylethanolamine†	14	49·5	5·8
Dipalmitoylphosphatidylethanolamine*	16	63	8·7
Dimyristoylphosphatidylserine‡	14	36	7·0
Dipalmitoylphosphatidylserine‡	16	53	9·0
Dioleoylphosphatidylserine‡	18 two unsaturated	−7	7·5

* Mabrey & Sturtevant (1976).

† Vaughan & Keough (1974) (also contains data for compounds with small variations in head-group).

‡ Browning & Seelig (1980).

Transition temperatures T_t and enthalpies ΔH_t of fully-hydrated phospholipid bilayers. Changes in ionic strength of the aqueous phase or in the state of hydration (water content) affect the values given above. Such information is valuable in theoretical studies of the interaction forces between phospholipid molecules, and with the aqueous phase. Phospholipid bilayers with unsaturated chains have much lower transition temperatures, normally below 0 °C.

bilayers. X-ray scattering experiments on lipid bilayers are often complemented by differential scanning calorimetry studies (e.g. Mabrey & Sturtevant, 1976) which provide information about the heats of transition between different phases. As an example, chain melting transition temperatures T_t and enthalpies ΔH_t of some common phospholipid bilayers are given in Table 1.

Another technique which has provided very detailed information about lipid chains in a bilayer, particularly in the fluid phase, is nuclear magnetic resonance (NMR). The time scale of a NMR measurement is about 10^{-5} s. During that time a typical molecule assumes a large number of different conformations and can diffuse over a large distance ($\sim$ 500 Å). The NMR signal therefore reflects the average value of the measured quantity over this time scale and may be insensitive to some of the more rapidly changing motions of the system. The most important NMR experiments have been on samples in which the C—H bonds were deuterated at specific positions along the chains. Deuteron

quadrupole resonance was then used to measure an average orientation of the C—D bonds with respect to the direction of the magnetic field (Charvolin, Manneville & Deloche, 1973; Seelig & Niederberger, 1974; Stockton *et al.*, 1976). Such experiments are usually performed on oriented multilayer samples, with the magnetic field normal to the bilayers, and have provided detailed information on the variation of local order along the hydrocarbon chains of lipid molecules (Seelig & Seelig, 1974). An example of results of such measurements is shown in Fig. 2.1.

If the averaged motion of a chain segment is assumed to be cylindrically symmetrical, the splitting of the signal is proportional to the deuterium *order parameter*

$$S_{CD} = \langle 3/2 \cos^2\theta - \tfrac{1}{2} \rangle,$$

where θ is the angle between the C—D bond and the direction of the magnetic field. For an aligned sample, the segmental order parameter defined above varies between the values of $S_{CD} = -\frac{1}{2}$, when a chain is in the all-*trans* state, and $S_{CD} = 0$ for a fully disordered (isotropically tumbling) segment. The most interesting feature of NMR results on lipid chain order in bilayer membranes is that the order parameter remains relatively constant along most of the chain, decreasing only near the end of the chain. This phenomenon has been observed in a variety of different lipid bilayers, e.g. neutral lecithin and phosphatidylethanolamine, charged phosphatidylserine, and *Acholeplasma laidlawii* lipids, and appears to be a general property of lipid chains in membranes (Seelig & Browning, 1978).

NMR measurements also indicate that the two chains of a phospholipid molecule in a fluid bilayer, i.e. at $T > T_t$, are not completely equivalent: the first two segments of one of the two chains are oriented almost parallel to the bilayer surface. It is interesting that this conformation is similar to that measured in the crystalline state (see Fig. 2.2 below). Thus one of the chains penetrates less deeply into the bilayer; this difference manifests itself once again in the terminal region of the chains where the NMR splittings of the last segments of chains 1 and 2 again show small differences.

The conventional description of NMR results in terms of a *single* order parameter does not make full use of the information potentially available. The average orientation of any C—D bond, measured by a quadrupole splitting of the resonance line, actually results from two physically distinct types of motion (Bos & Doane, 1978). First, the whole molecule can

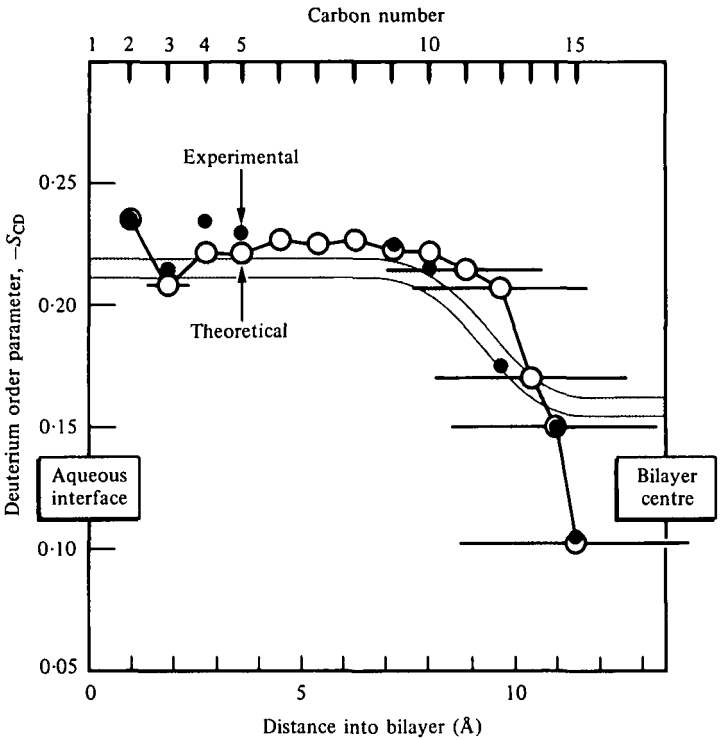

Fig. 2.1. Measured deuterium order parameter along a dipalmitoyl-lecithin chain in a bilayer (filled circles). The terminal 16th carbon was not measured. The theoretical profile (open circles) depends on the assumed value of the lateral pressure acting on the lipid chains (set to 18·5 dyn/cm in this example – see also Fig. 4.1). Note that the mean positions of the carbon atoms (top axis and open circles) are not linear with the distance into the bilayer (bottom axis). The horizontal bars indicate the standard deviation of the spatial motion of each carbon atom about its mean position, and the shaded strip shows the *mean* order parameter *along the bilayer*. Adapted from Seelig and Seelig (1974), Schindler & Seelig (1975), and Gruen (1980). Very similar deuterium order parameter profiles have been obtained for lipids in *A. laidlawii* membranes (Stockton *et al.* 1977).

change orientation with respect to the direction of the magnetic field by moving within or along the bilayer. Such reorientations of the molecule as a whole can be specified by reference to a molecular frame, defined with respect to some suitable molecular axis. Second, since a molecule is flexible, an individual C—D bond can change orientation with respect to the molecular frame. One can therefore define separate order parameters describing respectively the motion of the molecular frame, and the segmental motion with respect to that frame.

Lipid molecules are not axially symmetrical, and motions of the molecular

frame can be anisotropic or biased. For example, if we identify the long axis of the molecule with the z direction of the molecular frame, a biased motion could then mean that rocking of the molecule about the x-axis of the molecular frame is more pronounced than rocking about the y-axis. Some very recent NMR data on phospholipid chain motion (Vaz & Doane, 1980) and head-group motion (Vaz, Doane & Neubert, 1979) in bilayers indicate biased motions for the molecules as a whole as well as deviations of the chain motion from cylindrical symmetry. However, the quantitative analysis in the cited articles assumed that terms relating to the molecular motion and terms relating to segmental conformational changes can be averaged separately, i.e. that the two motions are independent. In a flexible lipid molecule, conformational changes will be strongly correlated with the motion of the whole molecule, so that the quantitative results of Vaz & Doane (1980) and Vaz *et al.* (1979) must be treated with caution.

Other experimental methods which have also provided useful information about the physical state of lipid chains in bilayers include electron spin resonance (ESR), Raman spectroscopy and fluorescence anisotropy. In particular, ESR studies with spin labels in both pure and mixed lipid bilayers, as well as in lipid-protein membranes, led to such discoveries as flip-flop, boundary lipids, chain tilting and lateral phase separations, discussed later. For a critical review of spin labels in membranes see Schreier, Polnaszek & Smith (1978). The sensitivity attainable with ESR is much higher than with NMR, and much faster motions (down to 10^{-10} s) can be monitored. Details of motional anisotropy of molecules can also be measured, as can the motional correlation times and order parameters associated with these motions (Shimoyama, Eriksson & Ehrenberg, 1978). However, both ESR and fluoresence spectroscopy suffer from the perturbing effects that the probes have on their local environment, and the data often reflect more on the restricted motions of the probes than on the molecules to which they are attached. Nevertheless, spin labelled probes can be relied upon to give trends if not always quantitatively reliable results, and will remain a powerful tool for probing a wide variety of membrane-associated phenomena.

Can we understand the basic features of lipid chain ordering within bilayer membranes? A substantial number of theoretical papers has addressed different aspects of that question. The phase transition between the liquid crystalline fluid phase and the rigid frozen phase has recently been reviewed by Nagle (1980). A statistical mechanics model of lipid chains in bilayer membranes has to incorporate the

following basic features of the system: (i) each chain may assume any of a large number of available rotational isomeric states, and (ii) the system is dense, and there is a strong mutual interaction between neighbouring chains. The problem is obviously very complicated, and approximate treatment has become inevitable.

Two early models of lipid chains in bilayers (Nagle, 1973; Marčelja, 1974*b*) still represent the two basic theoretically tractable options. The model of Nagle (1973) starts from the observation that the dominant interaction between the chains is a steric hard-core repulsion. An effective Hamiltonian of the system is written as

$$H = H_{\text{steric}} + H_{\text{rot}} + H_{\text{att}}.$$

The emphasis in this model is on an exact treatment of the steric repulsion and internal energy due to rotational isomerism; the contribution of the softer attractive interaction may be approximated without introduction of large errors. The calculation of the thermodynamic properties of the chains is carried out by restriction to a two-dimensional system, where all the possible chain conformations are in a plane. Such a system can then be identified with the exactly solvable planar model of dimers. The system is thus reduced to a solvable form while still retaining the exact treatment of the dominant repulsive interaction. The penalty incurred in following this procedure is the drastic simplification of interacting lipid chains to the two-dimensional schematic model.

The second approach (Marčelja, 1974*b*) is based on the similarity of a lipid bilayer interior to the more familiar liquid crystal systems. In liquid crystals, the orientational ordering can be relatively well described within a molecular field approximation. Anisotropic steric repulsion and van der Waals attraction between a molecule and its neighbours is replaced by a single effective interaction potential of the form

$$E = V_0\phi(\tfrac{3}{2}\cos^2\theta - \tfrac{1}{2}),$$

where ϕ is a self-consistently determined strength of the molecular field, θ is the molecular orientation with respect to the direction of the molecular field and V_0 is a coupling constant. This form is just the first non-vanishing term in the expansion of the orientational dependence of the molecular energy in terms of Legendre polynomials.

It can only describe orientational ordering in the system. The molecular field theory represents a serious over-simplification of intermolecular interactions, and in particular of steric repulsion. Nevertheless, experience from liquid crystal studies indicates that in a *fluid state*, the effective orientational ordering energy of a molecule is fairly well described by the above equation. In applying the molecular field approximation to lipid chains, contributions from each segment are included separately. The problem of interacting chains is then replaced by the single chain in a molecular field. The advantage of the method is that all conformations of a single chain can be exactly enumerated on a computer, or simplified using further approximations.

Both theoretical approaches have their strong and weak sides. The exact modelling of Nagle gives an insight into the detailed nature of the freezing phase transition, where molecular field approximations must fail. In a fluid phase, however, the molecular field approach is reasonable, and provides a much more accurate description of chains in real bilayers than the highly simplified and schematic exactly solvable models. Such theoretical modelling has resulted in new insight into the physical state of lipid chains in a bilayer. Comparison of computed results with order parameter measurements of Seelig & Niederberger (1974) has shown that lipid chains in a bilayer are more extended (i.e. have higher order) than would be expected for an isolated system of hydrocarbon chains (Fig. 2.1). This indicates that the chains are subject to a lateral pressure (Marčelja, 1974*b*), which is a consequence of hydrophobic and head-group interactions occurring at the aqueous interfaces, discussed below.

Another interesting conclusion has been reached by Gruen (1980), who computed the actual *distance*-dependence of the molecular order across a bilayer. Since lipid chains in the central region of a bilayer are highly mobile, and sample much of the bilayer interior during their motion, the strong decrease of order near the end of a lipid chain has only a relatively small influence on the variation of molecular order with distance across the bilayer (Fig. 2.1). The computed electron density profiles are also in good agreement with the corresponding X-ray measurements.

Solutions of exact models (Nagle, 1973) have further explained why the temperature dependence of such physical parameters as specific heat and lateral compressibility near the melting transition of lipid chains are not symmetrical about T_t. Below the melting temperature, conformational

changes of any chain are prevented by strong steric interactions with its neighbours; but above the transition, chains are relatively free and therefore more sensitive to temperature changes as the system is cooled towards freezing. This novel type of phase transition appears as a first-order phase transition below the freezing temperature and as a second-order transition above that temperature. Finally, theoretical research on lipid chains in bilayers has led to some new results in statistical physics. Nagle (1975) has discovered that the introduction of lateral pressure-area variables leads to an identification of a general non-local order parameter in the Kasteleyn model of dimers on a planar honeycomb lattice. The behaviour of a homologous series of thermotropic liquid crystals has also been successfully described by analogy with work on bilayer membranes (Marčelja, 1974*a*).

B. *Head-group structure*

Once the lipid chain interior of bilayers became fairly well characterized, the experimental attention concentrated more on investigations of the polar head regions. The major advances in elucidating the structure of lipid head-groups in bilayers have again been achieved using the techniques of X-ray and neutron scattering and nuclear magnetic resonance.

A complete determination of the crystal structure of a phospholipid (phosphatidylethanolamine: acetic acid) has been reported by Hitchcock *et al.* (1974). The conformation of a single molecule is shown in Fig. 2.2. The crystal structure of dimyristoyl lecithin dihydrate has been determined by Pearson & Pascher (1979), as has the structure of two related compounds, glycerophosphocholine (Sundaralingam, 1972) and 1-lauroyl-propanediol-3-phosphorylcholine (Hauser, Pascher & Sundell, 1980*a*). Such crystallographic work has been very important in understanding the molecular conformations of lipid polar heads in bilayers since NMR studies of the molecular packing of phosphatidylethanolamine and lecithin (Seelig, Gally & Wohlgemuth, 1977) show that the conformation of the crystal structure is retained *on average* in bilayers. This evidence is further corroborated by neutron scattering data (e.g. Büldt *et al.* 1978). A folded structure is stabilized in ethanolamines by a strong intramolecular hydrogen bond and in cholines by an electrostatic interaction between the phosphate group and the cationic trimethylammonium head (Pullman & Berthod, 1974). The zwitterion dipoles of these head-groups are mainly oriented parallel to the bilayer surface. In phosphatidylethanolamine the NH...OP intermolecular hydrogen bond network – characteristic of the crystalline structure –

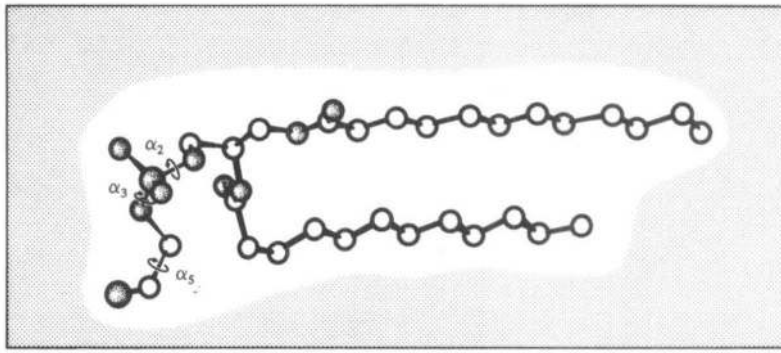

Fig. 2.2. The molecular conformation of 1,2 dilauroyl-DL-phosphatidylethanolamine in crystals with acetic acid. Redrawn from Hitchcock *et al.* (1974).

must therefore be retained to some extent in hydrated bilayers despite the fact that NMR data indicate rapid rotation of the molecules about their long axes, and increased motions of the head-groups. Indeed, fully hydrated phosphatidylethanolamine below T_t has the same head-group area of $\sim 39 \text{Å}^2$ as in the crystal state (Harlos, 1978), which increases only slightly above this value above the transition in inverted micellar phases.

In both lecithin and phosphatidylethanolamine bilayers NMR results are consistent with rapid transitions between two enantiomeric conformations of the head-group. The difference in conformation of phosphatidylethanolamine and lecithin head-groups is mainly restricted to a change in the sign of gauche states associated with the torsion angles, α_2, α_3, α_5 (see Fig. 2.2). The gauche sequence is $g^{\pm}g^{\pm}g^{\pm}$ in phosphatidylethanolamine and $g^{\pm}g^{\pm}g^{\mp}$ in lecithins. The motion is most restricted in the glycerol region and increases away from it, both in the direction of the aqueous phase and into the bilayer interior.

More recently, NMR studies have been performed on bilayers of phosphatidylserine (Browning & Seelig, 1980). While the average conformation of the chains and the glycerol backbone was found to be similar to other phospholipids, the data indicate a relatively rigid structure of the phosphatidylserine head-group. However, the motion of this head-group increased on dilution with lecithin, which indicates that head-group structure may not be considered as an intrinsic property of a particular lipid but rather as one that depends on the

interactions with neighbouring head-groups. An analogous conclusion applies to lipid chain motion, as is implicit in the earlier discussions.

In summary, recent experiments have found that the *average* structure of phospholipid head-groups in bilayers is similar to that in the crystalline states, which is also the quantum mechanical conformation of lowest energy (Pullman, Berthod & Gresh, 1975). A detailed description of head-group conformations and motion is, however, very involved. Taken alone, simple geometrical interpretations (in terms of discrete jumps between different conformational states) or simple motional models (in terms of free rotations about certain bonds) are not sufficient to account for the data (Browning & Seelig, 1980). Molecular rotation is likely to be strongly biased (Vaz *et al.*, 1979). Different segments of a head-group take part in a complex motion, spending more time in states with lower conformational energy. A more sophisticated combination of geometrical and motional considerations will be necessary to provide a detailed description of all the available data.

C. *Interactions at the bilayer/aqueous phase interface*

The hydrophilic head-groups of lipids in bilayers are subject to strong interactions with each other, with the surrounding aqueous medium, and with nearby bilayers, while being restricted to remain at the bilayer water interface by the hydrocarbon chains. All these interactions are interdependent, and we shall now consider them in turn, and their combined influence on bilayer structure.

(i) *Steric repulsion.* A strong force which acts only when molecules are in or near contact, steric repulsion is well understood. Since steric interaction depends only on the size of the molecules or molecular groups which are involved, it is accurately modelled by hard, impenetrable particles, e.g. hard spheres, discs or cylinders (Jacobs, Hudson & Andersen, 1975). The effect of hydration in determining the effective head-group size is, however, not well understood. We return to this matter below.

(ii) *Electrostatic interactions.* Ionic or zwitterionic charges on polar heads are subject to mutual interactions, interactions with ions or counterions in the aqueous phase, and interactions with dipole moments associated with water molecules. Electrostatic interactions between charged species and surfaces close together in aqueous solutions are not easily modelled. This fact is evident from the difficulties encountered in the formulation of an accurate theory of aqueous ionic solutions, and

is a central problem in liquid state chemistry and colloid science. By contrast, the surface charge density σ and potential ψ (as opposed to the forces) of ionizable membrane surfaces *are* relatively accurately described using the continuous Gouy–Chapman theory of the diffuse double layer with the added assumption of specific ion adsorption (Eisenberg *et al.*, 1979). The precise nature of the specific adsorption does not enter into the theory. Rather, it is assumed that the negative surface charge density can be described by an adsorption isotherm, e.g. of the Langmuir type:

$$\sigma = \sigma^{\max}/(1+KC_0),$$

where C_0 is the concentration of cations at the membrane surface given by diffuse double-layer theory, and K is an adjustable association constant.

The surface potential of membranes is normally measured using a charged fluorescent probe which adsorbs at the membrane–solution interface. The quantum yield of an adsorbed probe molecule is thereby increased, allowing for a determination of the surface concentration and consequently of the surface potential. Measurements of the electrophoretic mobilities of phospholipid vesicles also provide estimates of surface potentials – i.e. the potential at the hydrodynamic plane of shear (the ζ potential). Most measured values for the surface potentials of anionic phospholipid bilayers and vesicles lie in the range 60–90 mV (Eisenberg *et al.*, 1979). Electrophoretically determined ζ potentials are always smaller, indicating that the plane of shear is a few Ångstroms away from the interface where the ψ potential is measured.

It is somewhat intriguing that the measured surface potentials of a great variety of lipid bilayers, micelles, anionic (and cationic) soap films, and even charged mineral surfaces such as silica and mica, all appear to have potentials in the range 60–90 mV irrespective of the type of 1:1 electrolyte in the aqueous solution, and over a wide range of electrolyte concentrations even when the pH is well above the pK. This important observation implies that lipid bilayer surfaces possess a substantially lower effective surface charge density than would be expected for a fully-ionized surface. Such effects lead to smaller electrostatic repulsive forces between two charged bilayers than theoretically expected (Fig. 2.3) though the force law itself is still well described by double-layer theory except at distances below 30 Å (Cowley *et al.* 1978).

Since it is at such small separations that charged and zwitterionic head-groups interact *within* a bilayer it is not surprising that current theories cannot successfully account for these short-range interactions. The main problem arises from the existence of hydration shells (bound water) which give rise to their own repulsive force both between bilayers and between adjacent head-groups within a bilayer. The counterions near the charged interface compete with these water molecules, and their local distribution and interaction is no longer given by the Gouy–Chapman theory. Even the familiar van der Waals forces are changed at short distances by the discrete molecular structure of the medium (Ninham, 1980). As a result of these difficulties, pressure–area (Π–A) curves of charged monolayers at different salt concentrations do not follow the predictions of continuous double-layer theory (Forsyth *et al.* 1977). An accurate description of electrostatic head-group interactions is still not available.

(iii) *Hydration forces.* In a very novel experiment, Le Neveu, Rand & Parsegian (1976) measured a strong repulsive force acting between egg lecithin bilayers at separations below ~ 30 Å. The force was measured in a multilayer system by following the variation of the osmotic pressure with bilayer separation. The result, which is reproduced in Fig. 2.3, shows that the force follows an approximately exponential law, with the osmotic pressure P being given by $P \approx 10^{11} . \exp(-d_w/\xi_0)$ dyn/cm^2, where d_w is the thickness of the water layer separating individual bilayers, and $\xi_0 \simeq 2$ Å. It should also be noted that as the osmotic pressure is increased, the bilayers respond to their strong mutual repulsion by a lateral compression, thereby increasing their thickness and hence slowing down the decrease in d_w– an effect which highlights the interdependence of interbilayer and intrabilayer interactions.

It can be readily shown that this very strong force cannot be explained by a common double-layer or dipolar repulsion (indeed, the force between two zwitterionic surfaces should be attractive). The measured force was tentatively identified as a 'hydration' force on the basis of very general arguments (Marčelja & Radić, 1976) whereby a perturbation in the ordering of water molecules near a bilayer surface could be expected (within a continuum theory) to lead to an exponential repulsive law. Of course, when the continuum approximation is abolished, molecular structure should be superimposed on the smooth exponential dependence. The measured characteristic decay length of 2·6 Å

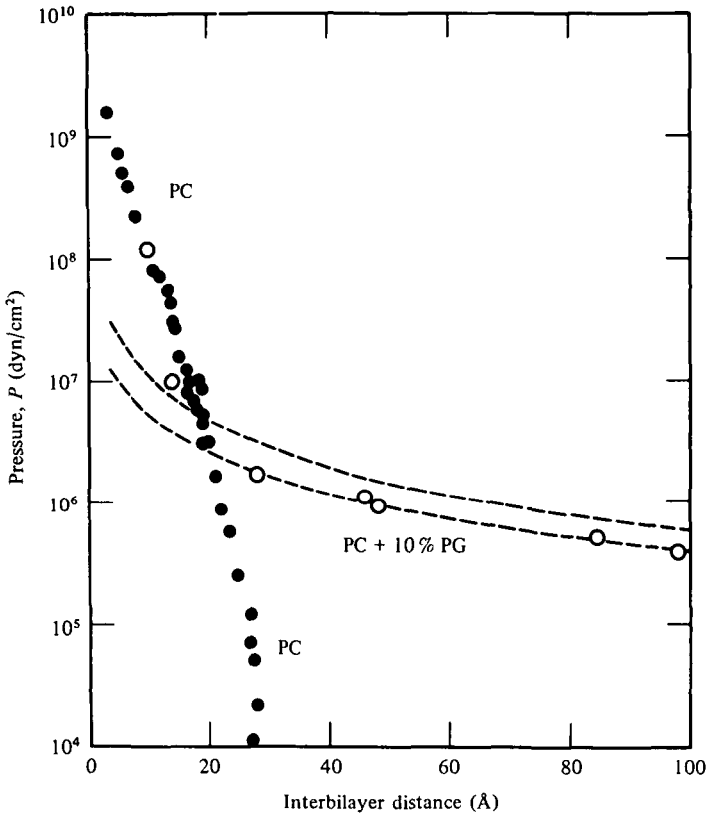

Fig. 2.3. Measurement of repulsion between egg lecithin, PC (●) and lecithin − 10 mole % phosphatidylglycerol, PC + 10 % PG (○) bilayers in water. With addition of charged phosphatidylglycerol the electrostatic repulsion dominates the interaction at interbilayer distances larger than about 25 Å. Below 25 Å repulsive hydration forces dominate. The theoretical electrostatic repulsion for fully ionized phosphatidylglycerol is shown by the upper curve. The experimental points, however, agree with theory for ~ 50 % ionization (lower curve). Redrawn from Cowley *et al.* (1978) and Parsegian *et al.* (1979).

(Parsegian, Fuller & Rand, 1979) is of the order of the molecular diameter of water, while the energy parameter in front of the exponential has the order of magnitude of the hydrogen bond energy in water.

Subsequent measurements of the repulsive force between charged bilayers are in agreement with the assumption that the strong repulsion originates from changes in water structuring near the bilayer surface (Cowley *et al.* 1978). A distinct region of double-layer repulsion, which

depends on the surface charge density alone is seen to change at smaller separation into a stronger repulsive law independent of the surface charge density. Such short-range repulsive forces are likely to exist between other phospholipid bilayers and are responsible for their initial swelling in water. The nature of changes in water structure near a bilayer surface are not well known. Several nuclear magnetic resonance experiments investigating the problem (e.g. Finer & Darke, 1974) have found that up to 20 water molecules per one lecithin molecule are to some extent affected by contact with the bilayer surface. Several types of 'bound' water could be distinguished. Calorimetric measurements (Ladbrooke & Chapman, 1969) found that about 10 water molecules per one lecithin molecule do not undergo freezing at 0 °C. It is hoped that a new generation of NMR experiments will lead to more detailed knowledge about the positional and orientational order of water at bilayer surfaces, and further work on force measurements will also be needed in order to establish whether the motions of the head-groups also make a steric contribution to these short-range repulsive forces.

(iv) *Interdependence of bilayer-associated interactions.* We have already seen how interbilayer and intrabilayer forces can have an effect on each other. In general all bilayer interactions – so far discussed largely in isolation – are interdependent, and we shall now review some of the more interesting phenomena that arise as a consequence of this interdependence.

One well-known effect of head-group interactions is the so-called pretransition which is seen for example in lecithin bilayers at a temperature about 10 degrees below the main chain freezing transition. Compared to the main transition, where the change in enthalpy is $\Delta H \simeq 9$ kcal/mole, the pretransition with $\Delta H \simeq 1$–2 kcal/mole is much weaker. At temperatures between the main transition and the pretransition, lipid chains are tilted with respect to the bilayer which itself is distorted by a periodic ripple (Janiak, Small & Shipley, 1976). Below the pretransition, the periodic ripple vanishes but the tilt of the chains gradually increases. The rippling effect is not easily understood (Doniach, 1979), but the tilting of the chains may be safely attributed to head group repulsion in lecithin and some other lipids (Jähnig *et al.* 1979): in a tilted configuration the lipid chains can remain in close contact while the area they occupy is larger than for perpendicular chains and so they are able to accommodate larger (or more repulsive) head-groups at the bilayer surface. Head-group repulsion is obviously

related to the molecular size and degree of hydration. Thus lecithin bilayers with relatively large head-groups and large hydration show significant tilt, while in phosphatidylethanolamine bilayers where the head-group and hydration is significantly smaller the chains remain in the perpendicular orientation (McIntosh, 1980). Consistent with this interpretation is the finding that at low water content (less hydration) lecithin bilayers do not undergo the pretransition and the tilt angle is at the same time reduced.

Similar effects are found with anionic lipid bilayers where the head-group interactions can now be modified by salt and pH. With increasing ionization (increased head-group repulsion) the tilt angle of chains in dihexadecylphosphatidic acid bilayers increases significantly (Jähnig *et al.* 1979). The lipid chain phase transition temperature and width is also affected when the head-group charge is varied by pH titration or addition of divalent cations (Träuble *et al.* 1976; Jacobson & Papahadjopoulos, 1975; MacDonald, Simon & Baer, 1976).

Head-group interactions within a bilayer are also correlated with the forces between different bilayers. This is to be expected since in both cases the same molecular groups are involved. Thus the large hydrated lecithin head-groups lead to a large surface area per lipid as well as significant swelling of fully-hydrated lecithin multilayers. By contrast, in phosphatidylethanolamine bilayers the head-group repulsion is much less, which leads to a smaller surface area per lipid as well as to a much reduced swelling in fully-hydrated multilayers, viz. 13 Å compared to about 27 Å for lecithin (Rand, Tinker & Fast, 1971; Harlos, 1978). Analogous correlations occur with anionic lipid bilayers where in general a decrease in pH or addition of divalent cations reduces the electrostatic head-group repulsion and hence the surface area per lipid, and leads to adhesion of vesicles or reduced swelling of multilayer phases (Papahadjopoulos & Miller, 1967; Papahadjopoulos *et al.* 1975).

The interesting effect of increased surface charge on broadening the chain phase transitions in anionic lipids has been interpreted as a consequence of domain formation in the vicinity of the phase transition temperature (Forsyth *et al.* 1977): near the transition temperature, it is favourable for the system to break up into fluid and solid domains in order to lower the electrostatic free energy associated with the diffuse double-layer.

We should also mention that valuable insight into the interactions governing bilayer structure can be obtained from corresponding studies of surface monolayers. Such work has an importance of its own since biological

monolayers do exist – at the surfaces of lungs, for example (Bangham, Morley & Phillips, 1979). The advantage of monolayer studies is that the lateral pressure can be externally varied, thus providing information which is not available from bilayer experiments. This is particularly important in studying the effect of pH and salt concentration on head-group interactions. The quantitative correspondence between bilayer and monolayer results is however, not readily obtainable. The question is usually formulated as a problem of finding an external surface pressure where monolayer and bilayer behaviour is equivalent. But it is not obvious that for any surface pressure the bilayer/monolayer equivalence holds at all temperatures. For example, changes in the area of a surface monolayer involve changes at the hydrocarbon/air interface which are not known, and may prevent quantitative comparison over a range of temperatures. However, at any particular temperature, a monolayer and a bilayer should be roughly equivalent at a surface pressure where their surface areas are equal.

In the final part of this section we have seen how the complex interactions associated with lipid chains and head-groups are interdependent. This interdependence can be seen as arising from the necessity of largely incompressible lipid molecules to simply pack together (cf. chain tilting). In the following sections we shall quantify these interactions and the molecular packing constraints that restrict them, and proceed with a detailed review of the thermodynamic principles of lipid self-assembly. This may appear somewhat contradictory, having just stressed the complexity of these interactions. However, a detailed knowledge of the forces involved is not needed! By analogy, the van der Waals equation of state contains no information on the origin and nature of the intermolecular forces, and yet provides a very satisfactory description of gas–liquid phase behaviour. Indeed, when van der Waals in 1873 proposed his now famous equation he knew nothing about what we now call van der Waals forces.

III. Thermodynamic principles of aggregation

A. *Introduction*

This section will be concerned with the thermodynamics of self-assembly in general and of amphiphilic molecules such as lipids in particular. The self-assembly of lipid molecules into well-defined structures such as micelles and bilayers is commonly ascribed to the favourable hydrophobic interaction in the aggregated phase; i.e. that the free energy per molecule in the aggregated state is less than that in

the dispersed state. This, while true, does not go to the heart of the matter, and leaves unanswered two crucial questions:

(i) Of all the possible structures that lipids can aggregate into, why do some form micelles (e.g. lysolecithin), some bilayers (e.g. lecithin), while others form only inverted micellar structures or precipitate out of solution? In order to answer this question it is obvious that not only must it be demonstrated that the free energy in the aggregated state is less than that in the dispersed state, but that it is also less than in any other aggregated state. Clearly, theoretical considerations of self-assembly should not be restricted to only one type of structure, e.g. bilayer, but must embrace all the alternatives. The need for a general theoretical framework is further highlighted by noting that different lipid structures often coexist in solution, and that they can be transformed from one type to another (e.g. micellar↔lamellar) by changing the lipid concentration or temperature (Luzzati & Tardieu, 1974).

(ii) What is the role of translational entropy in self-aggregation? The importance of entropy is usually overlooked in models which treat membranes as elastic sheets or as assemblies of purely mechanical components. However, it is well to remember that it is entropy that determines the concentration at which aggregates form, and that entropy is also involved in determining which structure – out of a number of possible candidates – is thermodynamically preferred. A further consequence that emerges from a consideration of the entropic contribution to self-assembly is that lipid and other macro-molecular structures may not be considered as separate entities, or possessing properties independent of their surroundings, with which they are in dynamic equilibrium. Thus a membrane in an aqueous solution depleted of lipids must be inherently less stable than one immersed in a lipid-rich medium. The former will simply evaporate away with time, since there will be no lipids coming in to replace those leaving due to their thermal motion. We return to this matter later in a discussion of lipid exchange.

B. *Thermodynamics of self-assembly*

Our first concern will be to establish the thermodynamic equations of self-assembly. The literature is voluminous and often confusing; the most rigorous statement of the problem being that of Hall & Pethica (1967) based on Hill's (1964) classic book on small system thermodynamics. We shall follow the approach of Tanford (1973) for micelles

which was later extended to larger lipid aggregates such as vesicles and bilayers by Israelachvili, Mitchell & Ninham (1976, 1977), and recently reviewed by Wennerström & Lindman (1979). The main point of departure from the earlier theories of Tanford and others was the recognition and elucidation of the role of molecular geometry in determining the structures that lipids or amphiphiles assemble into. These theoretical developments further showed that many of the physical properties of lipid aggregates, such as their shape, size, CMC, polydispersity, and even vesicle asymmetry (Carnie, Israelachvili & Pailthorpe, 1979) can be quantitatively described without requiring a detailed knowledge of the complex intermolecular forces between the polar head-groups and the hydrocarbon chains.

Conceptually, we shall reverse the order that theories of self-assembly have traditionally taken: thus rather than attempt first to quantify the highly complicated intermolecular forces and then to rationalize the results in terms of the known lipid structure, we shall first attempt to formulate the theory of aggregation in general thermodynamic terms (in the spirit of van der Waals' equation of state) that establishes the criteria which determine which structures will occur depending on the magnitude and sign of the interaction forces, the geometric properties of the molecules, lipid (or solute) concentration, temperature, etc. Once the basic thermodynamic principles of aggregation are established we then proceed in subsequent sections to compare the results with experimental data.

Equilibrium thermodynamics demands that the chemical potential of all identical molecules in a system of aggregated structures (micelles, bilayers, lipid or protein clusters in a membrane, etc.) will be the same. This may be expressed as

$$\mu_N^0 + \frac{kT}{N} ln(X_N/N) = \text{const.} \quad N = 1, 2, 3, \ldots, \tag{3.1}$$

where μ_N^0 is the mean free energy per molecule in aggregates of aggregation number N ($N = 1$ corresponding to isolated molecules in solution), X_N is the density of molecules incorporated into aggregates of number N, k is Boltzmann's constant and T is the temperature.

Depending on how the free energies μ_1^0, μ_N^0 are defined and measured, the dimensionless density X_N can be expressed as a *volume fraction* or *mole fraction* ((mole/litre)/55·5 for aqueous solutions). Equation (3.1) assumes ideal mixing, i.e. it is restricted to dilute systems where

interaggregate interactions can be ignored. The effect of interactions between bilayers and membranes will be considered later.

Equation (3.1), which can also be derived from the law of mass action (Alexander & Johnson, 1950), can be rewritten in the more convenient form

$$\begin{aligned} X_N &= N[(X_M/M)\exp(M(\mu_M^0-\mu_N^0)/kT)]^{N/M} \\ &= N(X_1\exp[(\mu_1^0-\mu_N^0)/kT])^N \end{aligned} \tag{3.2}$$

where M is any arbitrary reference state of aggregates (or monomers) with aggregation number M (or 1). Equation (3.2) together with the supplementary conservation relation

$$S = \sum_{N=1}^{\infty} X_N = \text{total solute concentration} \tag{3.3}$$

completely defines the system. Little more can be said about aggregated dispersions without spelling out the form and magnitude of μ_N^0 as a function of N. This matter will now be considered, and we proceed with some general remarks concerning μ_N^0.

C. *Conditions necessary for the formation of aggregates*

In the absence of any free energy contributions small aggregates are entropically favoured over larger ones. Thus for $\mu_N^0 = 0$, equation (3.2) becomes

$$X_N = NX_1^N, \tag{3.4}$$

i.e. since $X_1 < 1$, most of the molecules will be in the monomer state ($N = 1$). If μ_N^0 increases as N increases the above equations show that the occurrence of aggregates becomes even less probable. Thus the necessary condition for the existence of large stable aggregates is that μ_N^0 decreases as N increases, or that μ_N^0 has a minimum value at some value of N. Thus the functional variation of μ_N^0 with N determines the stability and, to a large extent, the physical properties of aggregates. Further since this variation may be a complex one it is clear that a number of structurally different populations may coexist and yet be in true thermodynamic equilibrium with each other (note that X_N in equation (3.2) is a distribution function and may peak at more than one value of N).

It is important to note that large aggregates need not form simply because there are large cohesive or binding energies between the molecules in the aggregated state. Such energies may make a large

bulk free energy contribution to μ_N^0, but if the molecules in different sized aggregates all experience the *same* interaction the value of μ_N^0 will remain constant per molecule in different aggregates so that $\mu_M^0 = \mu_N^0$ and equation (3.2) becomes equation (3.4) once again, and no large aggregates will form. However, depending on the *difference* in the interaction energy between the aggregated molecules and the solvent, the boundary energy contributions to μ_N^0 will always differ for aggregates of different sizes and shapes as a consequence of variations in these interactions, and it is this contribution that often largely determines how μ_N^0 varies with N. Thus the magnitude of the binding energies between the molecules in an aggregate is not a sufficient criterion for determining whether an aggregate will or will not form or what its size will be. The *variation* of the energy with size is what ultimately determines the properties of the aggregates, and we may anticipate that this is determined to a large extent by the shape of the aggregated structure. This shape depends, in turn, on the intermolecular forces and especially on the geometric packing properties (steric interactions) of the molecules. Simple molecules, such as small hydrocarbon molecules, may be treated as spherical so that their packing properties are fairly easy to model. But large amphiphilic molecules such as lipids and proteins require a more detailed consideration which takes into account their hydrophilic head-group area, their hydrophobic chain length and volume, and the interactions that affect these quantities. For lipid aggregates there are also bulk contributions to the free energy arising from different inter-chain and intra-chain interactions in different aggregates. However, before proceeding with an analysis of the packing and interaction properties of lipids, it is appropriate to continue with the formal thermodynamic development of the above equations.

We shall now consider the various functional forms of μ_N^0 for some typical aggregated structures, and by use of (3.2) and (3.3) describe their physical properties.

D. *Aggregate properties and their dependence on the interaction free energies*

(1) As mentioned above, aggregates will only form if μ_N^0 initially decreases with N. As will be seen the dependence of μ_N^0 on N is usually determined by the geometry of the aggregate. A form for μ_N^0 that

arises for some common lipid structures is (Israelachvili *et al.* 1976; Wennerström & Lindman, 1979):

$$\mu_N^0 = \mu_\infty^0 + \frac{\alpha kT}{N^P} \tag{3.5}$$

where α and P are constants. For such aggregates the mean free energy decreases asymptotically towards μ_∞^0 (the bulk free energy in an aggregate of infinite size) as N approaches infinity. The form of μ_N^0 as given by (3.5) is not generally likely to hold down to arbitrarily small N. However, if (3.5) does hold down to $N = 1$ the CMC is given approximately by†

$$\begin{aligned} \text{CMC} &= \exp\left[-(\mu_1^0 - \mu_N^0)/kT\right] \\ &= \exp\left[-\alpha\left(1 - \frac{1}{N^P}\right)\right] \\ &\approx \exp\left(-\alpha\right), \end{aligned} \tag{3.6}$$

which gives the concentration at which further addition of solute molecules results in the formation of more aggregates while leaving the monomer concentration unchanged (at the CMC value). That this is indeed so can be readily verified by noting that if the monomer concentration exceeds the CMC, substitution of (3.6) into (3.2) leads to a solute concentration X_N in aggregates greater than unity. Thus X_1 can never exceed the CMC.

We now consider two common forms of equation (3.5).

(i) $P = 1$.

$$\mu_N^0 = \mu_\infty^0 + \frac{\alpha kT}{N} \tag{3.7}$$

As will be seen such a dependence occurs for rod-like aggregates made up of linear chains of identical monomer units, and for spherical unilamellar vesicles in which the membranes bend elastically. When (3.7) is incorporated into equations (3.2) and (3.3) and solved (Israelachvili *et al.* 1976) the following results emerge:

The distribution of aggregates (X_N/N) is given by

$$(X_N/N) = \exp\left(-\alpha\right)\left[1 - \frac{1}{\sqrt{Se^\alpha}}\right]^N. \tag{3.8}$$

Thus the distribution is very broad, with (X_N/N) remaining constant for small N and decaying to zero only at large N ($N > \sqrt{Se^\alpha}$). The

† We shall retain the conventional use of the term *critical micelle concentration* or CMC to denote the critical aggregation concentration of *all* aggregated structures.

mean aggregation number, defined by $\bar{N} = \Sigma NX_N/\Sigma X_N = \Sigma NX_N/S$, is given by

$$\bar{N} = \sqrt{(1+4Se^{\alpha})}$$

$$\approx \begin{cases} 1+2Se^{\alpha} & \text{at low concentrations } S \qquad (3.9) \\ 2\sqrt{Se^{\alpha}} = 2\sqrt{(S/\text{CMC})} & \text{at high concentrations } S. \qquad (3.10) \end{cases}$$

Thus the aggregation number varies with the square root of the concentration S above the CMC. The actual magnitude of α, to which the aggregation number is very sensitive, depends of course on the intermolecular forces between the molecules or monomer units.

As an illustrative example we may consider an aggregated system composed of a linear chain of identical monomer units where the binding energy per unit is $-\alpha kT$ relative to that in the solvent. The free energy $N\mu_N^0$ of a linear aggregate composed of N such units is therefore (remembering that the terminal monomers are unbounded):

$$N\mu_N^0 = -(N-1)\alpha kT$$

i.e.

$$\mu_N^0 = -(1-1/N)\alpha kT = \mu_\infty^0 + \frac{\alpha kT}{N}$$

so that the mean free energy per molecule in such aggregates is given by (3.7). Thus if the binding free enegy is ~ 9 kcal/mole ($\alpha \approx 15$) the CMC will be $55\,e^{-15} \approx 2\times 10^{-5}$ M. For a unit of protein molecules of M.W. ≈ 110000 this corresponds to a CMC of about 1 mg/ml (these values apply to the assembly of the 6S tubulin dimers into microtubules (Timasheff, 1979)); and so at a concentration of ~ 10 mg/ml (ten times higher) the mean aggregation number should be $\bar{N} \approx 6$. For stronger binding energies the CMC will be lower, and the aggregation number higher at any given concentration. Such aggregation mechanisms occur in the self-assembly of certain lipids into rod-like micelles (Wennerström & Lindman, 1979) and may be relevant to the assembly of fibrous structures such as microtubules as well as to linear particle arrays often seen in membranes.

(ii) $P < 1$. If $P < 1$ a phase transition to large macroscopic aggregates occurs at the CMC (Israelachvili *et al.* 1976).

To see this intuitively we note that for a dispersion of aggregates each containing N molecules the translational entropy per molecule falls roughly as kT/N as N increases. Thus if the free energy falls more slowly than $1/N$ large aggregates will be favoured, i.e. for $P < 1$. For $P > 1$ only small aggregates are favoured.

As will be shown systems where $P < 1$ occur when oil is dissolved in water (and vice versa), and in the self-assembly of planar membranes. In general, all spherical or planar (disc-like) aggregates composed of

identical monomer units will have $P < 1$ ($P = \frac{1}{3}$ for spheres, $P = \frac{1}{2}$ for discs), compared with $P = 1$ for linear structures.

(2) In many cases μ_N^0 reaches a minimum value at some finite value of N (say $N = M$), or reaches a low value at $N = M$ and then remains almost constant for $N > M$.

Depending on the sharpness of the minimum in the free energy such a form for μ_N^0 results in monodisperse aggregates of mean aggregation number $\bar{N} \gtrsim M$, with a CMC occurring at (cf. equation (3.6))

$$\text{CMC} \approx \exp\left[-(\mu_1^0 - \mu_M^0)/kT\right]. \tag{3.11}$$

This is the common form traditionally used in the analysis of spherical micelles. If μ_N^0 has a minimum value at $N = M$ the variation of μ_N^0 about μ_M^0 can usually be expressed in the parabolic form:

$$\mu_N^0 - \mu_M^0 = \Lambda(\Delta N)^2, \tag{3.12}$$

where $\Delta N = (N - M)$. In this case equation (3.2) becomes

$$X_N = N\left\{\frac{X_M}{M}\exp\left[-M\Lambda(\Delta N)^2/kT\right]\right\}^{N/M} \tag{3.13}$$

and so the distribution in X_N will be near Gaussian with standard deviation in N given by

$$\sigma = \sqrt{(kT/2M\Lambda)}. \tag{3.14}$$

Systems which fall into the above thermodynamic category are spherical micelles and single bilayer vesicles.

E. *Formation of large aggregates: multilayers*

So far we have ignored inter-aggregate interactions; these cannot be ignored at high concentrations (low water content) where, especially for lipid dispersions, transitions to larger and more ordered mesophase structures are commonly observed. Both attractive and repulsive forces can lead to such phase transitions. We shall consider here only the transition of small lipid aggregates such as micelles and vesicles into extended multilayers. While the smaller aggregates are clearly favoured entropically, the larger aggregates could be energetically favourable due to boundary effects or if there is a force between the bilayers. The question is to establish how these two effects compete in determining which structure is formed at the CMC and at higher concentrations.

If M is the micelle/vesicle aggregation number and M the multi-

layer aggregation number ($\mathsf{M} \gg M$) then equating the chemical potentials of molecules in all the possible dispersed and aggregated states gives at equilibrium:

$$\mu_1^0 + kT \ln X_1 = \mu_M^0 + \frac{kT}{M} \ln \left(\frac{X_M}{M}\right) = \mu_{\mathsf{M}}^0 + \frac{kT}{\mathsf{M}} \ln \left(\frac{X_{\mathsf{M}}}{\mathsf{M}}\right)$$

or

$$\left(\frac{X_{\mathsf{M}}}{\mathsf{M}}\right) = \left\{\left(\frac{X_M}{M}\right) \exp\left[M(\mu_M^0 - \mu_{\mathsf{M}}^0)/kT\right]\right\}^{\mathsf{M}/M}. \qquad (3.15)$$

The concentration at which $X_{\mathsf{M}} = X_M$ is therefore

$$X_M = M \exp\left[-M(\mu_M^0 - \mu_{\mathsf{M}}^0)/kT\right]. \qquad (3.16)$$

Thus depending on M and the difference in the free energies $(\mu_M^0 - \mu_{\mathsf{M}}^0)$, which now includes the interactions between the molecules in the micellar and lamellar states, the latter may form spontaneously at the CMC, or – if X_M exceeds the CMC – at some higher concentration (while the background concentration of monomers and smaller aggregates remains unchanged). We may conveniently term such transitions first-order and second-order CMC's. If $\mathsf{M} \gg M$ the concentration at which large aggregates begin to form will be sharp and in all respects analogous to a first-order CMC. Note, too, that if we put $M = 1$ in (3.16) it reduces to (3.11) for the first-order CMC. Indeed, if we consider the smaller aggregates as if they behaved as monomers equations (3.15) and (3.16) are completely analogous to equations (3.2) and (3.11).

IV. Review of different aggregation processes

We now look at specific examples of various dilute solute and lipid dispersions which form aggregates in various solvents. While the thermodynamic equations of Section III will be used, the formulation of the interaction free energies in these examples will be less rigorous and, at times, obviously model-dependent.

A. *Immiscible liquids*

Consider the dispersion of, say, small hydrophobic molecules such as oil in water. For a given aggregation number N the aggregate of minimum free energy is a spherical droplet of radius R such that $Nv = \frac{4}{3}\pi R^3$ where v is the molecular volume per oil molecule. The free energy of

the sphere is given by $N\mu_\infty^0 + 4\pi R^2\gamma$, where μ_∞^0 is the bulk energy per molecule, and γ is the interfacial energy per unit area. Hence

$$\mu_N^0 = \mu_\infty^0 + \frac{4\pi R^2\gamma}{N} = \mu_\infty^0 + 4\pi\gamma\left(\frac{3v}{4\pi}\right)^{\frac{2}{3}} \Big/ N^{\frac{1}{3}} \tag{4.1}$$

Therefore

$$\mu_N^0 = \mu_\infty^0 + \alpha kT/N^{\frac{1}{3}}, \tag{4.2}$$

where

$$\alpha = 4\pi\gamma(3v/4\pi)^{\frac{2}{3}}/kT \approx 4\pi r^2\gamma/kT, \tag{4.3}$$

where r is the effective radius of the molecule.

From the analysis given in the previous section, the above form for μ_N^0 leads to the expectation that oil molecules when dispersed in water will remain as monomers up to the CMC given by equation (3.6), i.e.

$$\text{CMC} = [X_1] \approx \exp(-\alpha) \approx \exp(-4\pi r^2\gamma/kT) \tag{4.4}$$

and that, because of the $1/N^{\frac{1}{3}}$ dependence of μ_N^0, any additional molecules will separate out into a bulk oil phase, which may be considered simply as a very large aggregate! Small aggregates in the form of dimers, trimers, etc. will always be present: the concentration of solute molecules in such aggregates of radius R will be

$$\begin{aligned} X_N &= N\{X_1 \exp[(\mu_1^0 - \mu_N^0)/kT]\}^N \\ &= N(X_1 e^\alpha)^N \exp(-4\pi R^2\gamma/kT) \\ &\approx N \exp(-4\pi R^2\gamma/kT). \end{aligned} \tag{4.5}$$

Thus there will be very few aggregates of any appreciable size (i.e. $R > 2r$) for $\gamma > 10$ erg/cm^2. The CMC for such systems is generally referred to as the *solubility* of the solute, and α represents the free energy of transfer of a solute molecule from bulk into the solvent medium. A similar analysis applies to all immiscible liquids.

As examples we first consider the solubility of water in hydrocarbon liquids at 25 °C where $\gamma \approx 50$ erg/cm^2. (Finkelstein (1976) has concluded that H_2O and nonelectrolytes cross lipid bilayers as if their interiors behaved as ordinary oils.) For one water molecule we take the effective radius r as $r = 1{\cdot}34$ Å (Hermann, 1975). The solubility of water in hydrocarbon, given by (4.4), is then $[X_1^{H_2O}]_{hc} \approx 6.5 \times 10^{-2}$, which perhaps fortuitously compares well with the measured solubilities (Ostwald solubility coefficients) of $5{\cdot}5 - 8{\cdot}5 \times 10^{-2}$ for water in various alkanes (Schatzberg, 1963). Conversely we may use (4·4) to calculate the free energy of transfer of a methane molecule from water into bulk

hydrocarbon liquid. For methane $r = 1{\cdot}83$ Å (Hermann, 1975) and γ should be the same as above. Thus $\alpha = 4\pi r^2\gamma = 2{\cdot}1 \times 10^{-13}$ erg or 3·0 kcal/mole. This may be compared with the experimental value for the solubility or 'hydrophobic energy' of methane of $\sim 3{\cdot}5$ kcal/mole (Hermann, 1975).

B. *Hydrated ions*

An important extension of the previous case is to do with the occurrence of so-called 'ionophores' or hydrated ions in hydrophobic solvents and membrane interiors (MacDonald, 1976; Ashcroft & Coster, 1978).

The partitioning of small ions between water and liquid hydrocarbon is strongly one-sided with almost all the ions remaining in the aqueous phase. This is because the electrostatic free energy of an ion of radius r and charge e in a medium of dielectric constant ϵ is $e^2/2r\epsilon$ so that the change in energy per ion on going from water ($\epsilon_w \approx 80$) to a pure hydrocarbon phase ($\epsilon_{hc} \approx 2$) is

$$\Delta\mu^0 \simeq -\frac{e^2}{2r}\left(\frac{1}{\epsilon_w}-\frac{1}{\epsilon_{hc}}\right) \approx \frac{e^2}{2r\epsilon_{hc}}. \tag{4.6}$$

Thus for a typical ion of radius $r = 1$ Å we find $\Delta\mu^0 \approx 140\ kT \approx 80$ kcal/mole, and the partition coefficient is consequently very small, viz $\exp(-\Delta\mu^0/kT) \approx 10^{-60}$.

It is important to point out however that the hydrocarbon interior of hydrated phospholipid bilayers is much more polar than that of a pure alkane due to water penetration and the thinness of the hydrocarbon region (usually $\simeq 30$ Å). Indirect measurements of ϵ_{hc} inside hydrated phospholipid bilayers indicate a value well above 20 down to the C_5 position, and about 5·5 at the C_{12} position (Griffith, Dehlinger & Van, 1974). This would substantially reduce the activation energy given by equation (4.6). We shall assume that for bilayer interiors the effective dielectric constant ϵ_{hc} lies in the range 2–6.

But there is another reason why the above equation is grossly oversimplified: as pointed out by MacDonald (1976) an ion can enter a hydrocarbon phase surrounded by a shell of water molecules. Considering such an ionophore to have radius R the free energy change for the ion plus water molecules on transferring to the hydrocarbon phase is now

$$\Delta\mu^0 \simeq \frac{e^2}{2R\epsilon_{hc}} + 4\pi R^2\gamma, \tag{4.7}$$

where γ is the water–hydrocarbon interfacial energy ($\approx$ 50 erg/cm^2). The distribution of water molecules in hydrated single ions of water aggregation number N is now given by

$$X_N = N\{X_1^{H_2O} \exp \underset{\text{water}}{[(\mu_1^0 - \mu_N^0)/kT]}\}^N \{X_1^i \exp \underset{\text{ions}}{[(\mu_1^i - \mu_N^i)/kT]}\}, \quad (4.8)$$

where $X_1^{H_2O}$ is the concentration of water monomers in the hydrocarbon phase, given by $[X_1^{H_2O}]_{hc} = \exp(-\mu_1^0/kT)$ as before (cf. equation (4.4)); X_1^i is the concentration of ionic monomers in the hydrocarbon phase and μ_1^i their free energy, which are related to the ionic concentration in the aqueous phase $[X_1^i]_{H_2O}$ by $[X_1^i]_{hc} = [X_1^i]_{H_2O} \exp(-\mu_1^i/kT)$. Thus (4.8) reduces to

$$X_N = N[X_1^i]_{H_2O} \exp[-(e^2/2R\epsilon_{hc} + 4\pi R^2\gamma)/kT]. \quad (4.9)$$

The most probable ionophore radius R_0 is given by $\partial(X_N/N)/\partial R = 0$, i.e.

$$R_0 = (e^2/16\pi\epsilon_{hc}\gamma)^{\frac{1}{3}} = \begin{cases} 3{\cdot}6 \text{ Å} & \text{for } \epsilon_{hc} = 2{\cdot}0 \\ 2{\cdot}5 \text{ Å} & \text{for } \epsilon_{hc} = 6{\cdot}0 \end{cases}. \quad (4.10)$$

The distribution of ionophores therefore peaks at a radius close to 3 Å, corresponding to a significant hydration number. The energy at the minimum is now

$$\begin{aligned} \Delta\mu_{min}^0 &= 3e^2/4R_0\epsilon_{hc} = 12\pi R_0^2\gamma \\ &= (27\pi e^4\gamma/4\epsilon_{hc}^2)^{\frac{1}{3}} \simeq \begin{cases} 35 \text{ kcal/mole for } \epsilon_{hc} = 2{\cdot}0 \\ 17 \text{ kcal/mole for } \epsilon_{hc} = 6{\cdot}0 \end{cases} \end{aligned} \quad (4.11)$$

and the concentration of ionophores in the hydrocarbon phase is therefore

$$\begin{aligned} (X_N/N) &= [X_1^i]_{H_2O} \exp(-\Delta\mu_{min}^0/kT) \\ &\simeq \begin{cases} 10^{-26}[X_1^i]_{H_2O} & \text{for } \epsilon_{hc} = 2{\cdot}0 \\ 10^{-12}[X_1^i]_{H_2O} & \text{for } \epsilon_{hc} = 6.0. \end{cases} \end{aligned} \quad (4.12)$$

Note that equations (4.10) – (4.12) are independent of the size of the ions. Equation (4.12) may be compared with the value of

$$[X_1^i]_{hc} \exp(-e^2/2r\epsilon_{hc}kT) \approx \begin{cases} 10^{-61}[X_1^i]_{H_2O} & \text{for } \epsilon_{hc} = 2{\cdot}0 \\ 10^{-22}[X_1^i]_{H_2O} & \text{for } \epsilon_{hc} = 6{\cdot}0 \end{cases} \quad (4.13)$$

for isolated ions of radius 1 Å. Thus the equilibrium concentration of ionophores is many orders of magnitude greater than that of isolated

ions, and depends critically on the polarity of the hydrocarbon as reflected by the value of its dielectric constant ϵ_{hc}. In order to narrow the range of possible values for ϵ_{hc} we note that the ratio of the partition coefficients of Na^+ and H_2O in bilayers, as deduced from permeability studies (Hauser, Oldani & Phillips, 1973), is $\sim 10^{-11}$. Since $[X_1^{H_2O}]_{hc} \approx 6 \times 10^{-2}$, therefore $(X_N/N) \approx 6 \times 10^{-13}\ [X_1^i]_{H_2O}$. Comparing this with equation (4.12) leads once again to a value of $\epsilon_{hc} \approx 6{\cdot}0$ for the effective dielectric constant which determines the passage of ions through bilayers.

Ionophore size distributions should be Gaussian with a standard deviation in R of $\sim \sqrt{(kT/8\pi\gamma)} \approx 0{\cdot}6$ Å. This system does not have a CMC.

MacDonald (1976) has suggested that the above mechanism may account for the similar permeability that different ions have when traversing lipid membranes since their partitioning into the hydrocarbon phase, here given by (4.12), is independent of ion size.

The above analysis highlights the enormous sensitivity of ionophore concentration to local variations in the dielectric constant within a membrane – an effect that is bound to be important in permeability, flip-flop (see later), and membrane bioenergetics (Ashcroft & Coster, 1978).

C. *Lipid aggregates*

We first review the mechanisms that govern the self-assembly of fluid lipids into well-defined structures such as micelles, bilayers, etc. The major forces derive from the hydrophobic interaction between the hydrocarbon tails, which induces the molecules to associate, and the hydrophilic nature of the head-group, which imposes the opposite requirement that they remain in contact with water. These two interactions compete to give rise to the idea of two 'opposing forces' (Tanford, 1973) acting mainly in the interfacial region: the one tending to decrease and the other tending to increase the interfacial area a per molecule (the head-group area) exposed to the aqueous phase. The attractive interaction arises mainly from the attractive hydrophobic or interfacial tension forces acting at the *fluid* hydrocarbon–water interface and may be represented by a positive interfacial free energy per unit area of $\gamma \approx 50$ erg/cm^2 (Oldani *et al.* 1975; Hui *et al.* 1975). Thus the interfacial hydrophobic free energy contribution to μ_N^0 may be written as γa. The repulsive contributions are too complex and difficult to

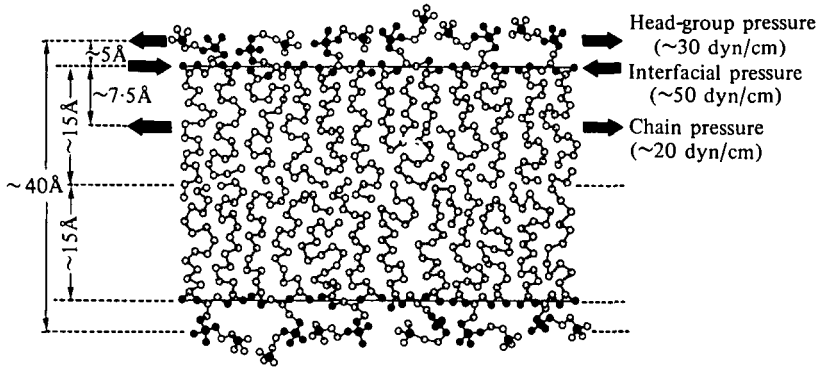

Fig. 4.1. Schematic representation of balance of forces in a phospholipid bilayer. The repulsive forces arising from head-group and chain interactions balance the attractive hydrophobic surface tension. The approximate value for the surface tension is 50 dyn/cm; the value of ~ 20 dyn/cm for the lateral chain pressure is based on the theoretical calculations of Marčelja (1974*b*) and Schindler and Seelig (1975). Note that the two repulsive force contributions are equivalent to a net force of ~ 50 dyn/cm effectively centred near the hydrocarbon–water interface (since $20 \times 7{\cdot}5 \approx 30 \times 5$). In general the attractive and repulsive forces do not act in the same plane, as discussed in the text.

formulate explicitly; these include the electrostatic (double-layer) repulsion between charged head groups, hydration forces, and steric head-group and chain interactions (these interactions were discussed in Section II). However, as in the two-dimensional van der Waals equation of state, we expect the first term in any energy expansion to be inversely proportional to the area a. The interfacial free energy per molecule in an aggregate may therefore be written, to first order, as

$$\mu_N^0 = \gamma a + C/a. \tag{4.14}$$

where C is a constant. We shall initially assume that both these forces act in the same plane (Fig. 4.1). The minimum free energy is therefore given when $\partial\mu_N^0/\partial a = 0$, i.e.

$$\mu_N^0\,(\min) = 2\gamma a_0, \tag{4.15}$$

where $a_0 = \sqrt{C/\gamma}$, a_0 will be referred to as the 'optimal surface area' per molecule defined at the hydrocarbon–water interface. The interfacial energy per molecule, equation (4.14), may now be expressed in the more convenient form

$$\mu_N^0 = 2\gamma a_0 + \frac{\gamma}{a}(a - a_0)^2 \tag{4.16}$$

in which the unknown constant C has been eliminated, so that μ_N^0 as a function of a is now in terms of the two known or measurable parameters γ and a_0. We see therefore how the concept of opposing forces leads to the concept of an 'optimal area' per head-group at which the total interaction energy per lipid molecule is a minimum.

The above three equations, while crude, nevertheless contain the essential features of inter-lipid interactions in micelles, bilayers and membranes. Thus equation (4.14) contains both the attractive γa *and* repulsive C/a contributions to the net interaction. This is essential and has often led to some confusion: a membrane or BLM cannot be considered to possess only a surface tension (or else it would not remain as a bilayer but curl up into a macroscopic spherical droplet). The observation that certain BLMs have a measurable surface tension or energy of $\sim$ 1 erg/cm^2 should not be construed as reflecting an inherent property of bilayers. Rather it represents the difference between the free energies of lipid molecules in the bilayer and in (probably inverted) micelles in the Plateau–Gibbs border (i.e. the bulk reservoir). The surface tension contribution exists, but it is opposed by the head-group and chain repulsive forces which together maintain the dispersed lipid structure. But why do the two opposing forces take such different forms? The repulsive forces arise from head-groups and chains constrained to interact in a plane (i.e. in two dimensions), and for such cases the first term in a virial expansion must follow a C/a dependence for the energy, or C/a^2 for the pressure, as found experimentally in monolayer studies of lecithin at the oil–water interface by Taylor, Mingins & Pethica (1976). The purely attractive surface tension contribution arises from the assumed liquid-like nature of the hydrocarbon–water interface and therefore has a simple γa dependence.

The above equations imply that the interaction energy between lipids has a minimum at a certain head-group area a_0, about which the energy varies parabolically, i.e. elastically. Indeed, the elastic compressibility modulus k may be readily obtained from (4.16), since by definition

$$\text{elastic energy} = \tfrac{1}{2}k\frac{(a-a_0)^2}{a} \qquad (4.17)$$

which immediately gives a value of $k \approx 2\gamma \approx 100$ erg/cm^2, in agreement with the value of 99 ± 22 (SD) dyn/cm (erg/cm^2) recently measured by Kwok, Evans & Hochmuth (1980) for the area compressibility modulus

of lecithin bilayers, and equal to the value of $\sim 10^2$ dyn/cm characteristic of cell membranes (Evans & Skalak, 1979).

Having established the equations that adequately describe the interaction between lipids within aggregates we have yet to establish which type of structure different lipids will assemble into. Geometric considerations must now be applied to determine the most favoured lipid structure in which the interaction energy μ_N^0 and translational entropy are together optimized according to equation (3.2). The shapes and sizes of molecules now enter the picture. The geometric or packing properties of lipids depend on their (measurable) area a_0, the volume v of their hydrocarbon chain, which will be assumed to be fluid and incompressible, and the *maximum* length that the chains can assume. We shall call this the maximum or 'critical chain length' l_c. This length sets a limit on how far the chains can extend; smaller extensions are allowed but further extensions are not – these being prevented by a sharp rise in free energy. The critical length l_c is a semi-empirical parameter, since it represents a somewhat vague cut-off distance beyond which hydrocarbon chains can no longer be considered as fluid. However, as may be expected it is of the same order, though somewhat less than, the fully-extended length of the chains (Tanford, 1973; Israelachvili *et al.* 1976, 1977).

Once the optimal surface area a_0, hydrocarbon chain volume v, and critical length l_c are specified for a given lipid – all these being measurable or estimable† – it is possible to determine the shape and size of the structure that the lipids can pack into whilst maintaining these parameters. It turns out that these can be satisfied by a great variety of different structures. However, it follows from Section III that since μ_N^0 is roughly the same for all these structures (since a_0 is the same) entropy will favour the structure with the smallest aggregation number, and this structure is unique! Larger structures will be entropically unfavoured while smaller structures, where packing constraints cause the surface area a to increase above a_0, will be energetically unfavoured.

It has been shown previously (Israelachvili *et al.* 1976) that for lipids of optimal area a_0, hydrocarbon volume v, and critical chain length l_c, the value of the dimensionless 'packing parameter' $v/a_0 l_c$ will determine whether they will form spherical micelles ($v/a_0 l_c < \frac{1}{3}$), non-spherical

† $v \approx (27{\cdot}4 + 26{\cdot}9n)$ Å^3 and $l \approx (1{\cdot}5 + 1{\cdot}265n)$ Å per saturated hydrocarbon chain with n carbon atoms (Tanford, 1972).

micelles ($\frac{1}{3} < v/a_0 l_c < \frac{1}{2}$), or bilayers ($\frac{1}{2} < v/a_0 l_c < 1$). Each of these structures corresponds to the minimum-sized aggregate in which *all* the lipids have minimum free energy, i.e. $a = a_0$. This, however, does not always guarantee that these structures will form since smaller structures with a higher μ_N^0 may be entropically more favourable. For example, when $v/a_0 l_c \gtrsim \frac{1}{2}$ large toroidal micelles are energetically favoured whereas smaller rod-like micelles with energetically unfavourable ends may be the thermodynamically favoured micellar phase. Thus, in general, we must consider all the possible structures even when μ_N^0 is not necessarily a minimum, and in most cases one finds that the thermodynamically favoured state is that in which the micelles are a bit smaller and the free energy μ_N^0 a bit larger than those for which the free energy is a minimum. Fig. 4.2 illustrates how the structures formed by some common lipids relate to their packing properties, and how these are affected by their ionic environment, temperature, unsaturation, etc., as will now be described.

D. *Spherical micelles*

For lipids to assemble into spherical micelles their optimal surface area a_0 must be sufficiently large and their hydrocarbon volume v sufficiently small such that the radius of the micelle will not exceed the critical chain length l_c. From simple geometry the micellar radius R will be given by

$$R = 3v/a_0 \leqslant l_c, \tag{4.18}$$

i.e. $v/a_0 l_c < \frac{1}{3}$ and the aggregation number will be

$$M = 4\pi R^3/3v = 4\pi R^2/a_0. \tag{4.19}$$

Examples of such micellar lipids are sodium dodecyl sulphate (NaDS) in water where experimentally, $M \approx 57$, $v = 350$ Å^3, and therefore $a_0 \approx 62$ Å^2, $R \approx l_c \approx 17$ Å. Note that such lipids can also pack into bilayers, but they do not self-assemble into bilayers because it is entropically unfavourable.

Most lipids that form spherical micelles have charged head-groups since this leads to large head-group areas a_0. Addition of salt partially screens the electrostatic head-group repulsion and thereby reduces a_0 leading to larger non-spherical micelles. For example, the aggregation number of NaDS micelles in 0·6 M NaCl is $\sim$ 1000 compared to $\sim$ 57 in water.

The size of spherical micelles is relatively insensitive to lipid concentration above the CMC, and the micelles are fairly monodisperse.

Lipid	Critical packing parameter $v/a_0 l_c$	Critical packing shape	Structures formed
Single-chained lipids (detergents) with large head-group areas: NaDS in low salt Some lysophospholipids	$< \frac{1}{3}$	Cone	Spherical micelles
Single-chained lipids with small head-group areas: NaDS in high salt Non ionic lipids lysolecithin	$\frac{1}{3} - \frac{1}{2}$	Truncated cone or wedge	Globular or cylindrical micelles
Double-chained lipids with large head-group areas, fluid chains: Lecithin, sphingomyelin Phosphatidylserine in water Phosphatidylglycerol Phosphatidylinositol Phosphatidic acid Disugardiglycerides Some single-chained lipids with very small (uncharged) head-groups.	$\frac{1}{2} - 1$	Truncated cone	Flexible bilayers Vesicles
Double-chained lipids with small head-group areas, anionic lipids in high salt, saturated frozen chains: Phosphatidylethanolamine, Phosphatidylserine + Ca^{2+}	~ 1	Cylinder	Planar bilayers
Double-chained lipids with small head-group areas, nonionic lipids, poly(cis) unsaturated chains, high T: Unsat phosphatidylethanolamine Cardiolipin + Ca^{2+} Phosphatidic acid + Ca^{2+} Monosugardiglycerides Cholesterol	> 1	Inverted truncated cone	Inverted micelles

Fig. 4.2. Dynamic packing properties of lipids and the structures they form The thermodynamically favoured structures are determined by the interplay of entropy which favours small structures, and packing constraints (characterized by the critical packing parameter) which energetically resist the packing of molecules into arbitrarily small-highly curved structures.

The standard deviation in the aggregation number is (substituting (4.16) into (3.12) – (3.14)):

$$\sigma = \sqrt{\left(\frac{9kT}{2\gamma a_0}\right)}\ \sqrt{M}. \qquad (4.20)$$

Thus for typical values of $\gamma \approx 50$ erg/cm^2, $a_0 \approx 60$ Å^2, we obtain

$$\sigma \approx \sqrt{M}, \tag{4.21}$$

i.e. for $M \approx 60$, $\sigma \approx 8$. The distribution or spread about M is thus fairly sharp.

E. *Non-spherical and rod-like micelles*

Those lipids which possess smaller head-group areas such that $\frac{1}{3} < v/a_0 l_c < \frac{1}{2}$ cannot pack into spherical micelles but can form cylindrical (rod-like) structures. Thus single-chained lipids possessing charged head-groups in high salt or those possessing uncharged (non-ionic or zwitterionic) head-groups fall into this category (e.g. NaDS in high salt, lysolecithin).

As discussed in Section III(D), rod-like aggregates have very unusual properties: they are large ($\bar{N} > 1000$) and polydisperse, and their mean aggregation number $\bar{N}$ is very sensitive to the total lipid concentration S and ionic strength.

For recent reviews of micelles see Fisher & Oakenfull (1977), and Wennerström & Lindman (1979).

F. *Bilayers*

Lipids that form bilayers are those which cannot pack into small micellar structures due to their small head group area a_0 or – as is more common – because their hydrocarbon chain region is too bulky to fit into such small aggregates while maintaining the surface area at its optimal value. For bilayer-forming lipids the value of $v/a_0 l_c$ must lie between $\frac{1}{2}$ and 1, and this requires that for the same head group area a_0 and chain length l_c their hydrocarbon volume v must be about twice that of micelle-forming lipids (for which $v/a_0 l_c < \frac{1}{2}$). Therefore lipids with two chains are likely to form bilayers, and indeed most of them do. However, the doubling of the chains also increases the hydrophobic energy of the lipids, and from (3.11) this drastically lowers their CMC (compare the CMCs of common micelle-forming lipids with 12–16 carbons, 10^{-2} to 10^{-3} M, with that of bilayer-forming lipids, $\sim 10^{-10}$ M). One important consequence of this large difference in the CMC is that the 'residence times' of lipids in micelles is much shorter than the residence times of lipids that form bilayers. Now the rate at which a lipid in an aggregate of number M will hop out into the aqueous solution is given by (cf. Aniansson *et al.* 1976, equation (50), etc., as applied to micelles):

$$\text{Rate} = \nu_c \exp\left[-(\mu_1^0 - \mu_M^0)/kT\right]. \tag{4.22}$$

where ν_c is the collision frequency of the lipid in the aggregate, and $\exp[-(\mu_1^0-\mu_M^0)/kT]$ the probability of finding the lipid molecule with higher energy than the hydrophobic energy barrier needed to take it out of the aggregate into solution Values of $\nu_c = 1/\tau_c$ should be close to typical rotational frequencies of lipids in aggregates. Thus the lifetime of a lipid τ in an aggregate in water may be written in the form

$$\tau = 1/\text{Rate} \approx \tau_c \exp[(\mu_1^0-\mu_M^0)/kT] \approx \frac{55\tau_c}{\text{CMC}}, \qquad (4.23)$$

where (3.11) has been used and where the CMC is now in mole/litre (hence the factor 55 in the numerator). Typical measured values for τ_c are $10^{-7}-10^{-9}$ s. Thus for micelles and bilayers we have

$$\tau \text{ (micelles)} \approx 55\times 10^{-8}/10^{-2} \sim 10^{-4} \text{ s}$$
$$\tau \text{ (bilayers)} \approx 55\times 10^{-8}/10^{-10} \sim 10^{+4} \text{ s}.$$

The above order of magnitude estimates are close to those that have been measured, both for the residence time of surfactant or detergent molecules in micelles where for $C_{12}-C_{16}$: $\tau \sim 10^{-3}-10^{-5}$ s (Fisher & Oakenfull, 1977; Wennerström & Lindman, 1979) and for the exchange or transfer rates of diacyl phospholipids and cholesterol between bilayers and vesicles, where $\tau \sim$ 2–24 hours, i.e. $\tau \sim 10^4-10^5$ s (Roseman & Thompson, 1980; Papahadjopoulos *et al.* 1976; Duckwitz-Peterlein, Eilenberger & Overath, 1977; Thilo, 1977; Haran & Shporer, 1977).

Thus whereas in micelles the residence time is very short, that in lipid bilayers is very long, though strictly these lifetimes are more a property of the lipid than of the structure. Thus lysolipids may be expected to have much shorter residence times in bilayers than double-chained lipids.

It is interesting to note that residence times of the order of hours for lipids in bilayers can be very similar to typical flip-flop rates (see Haran & Shporer, 1977, for example) so that in experimental studies these two processes may often be equally important. Indeed, flip-flop may be regarded as the inverse of membrane exchange, wherein the lipid hops into the bilayer rather than out of it. Were we to have better data on the energy needed to take a lipid head-group into the hydrocarbon interior (analogous to the hydrophobic energy barrier in lipid exchange) we would have a better understanding of flip-flop. Green, Fry & Blondin (1980) recently postulated that phospholipids have ionophoric capabili-

ties, and that they mediate the transport of solutes and ions across membranes. If a hydrated head-group is regarded as an ionophore, equation (4.11) shows how variable the activation energy for flip-flop may be. Nevertheless, putting $(\mu_1^0 - \mu_{\ddagger}^0) \approx 17$ kcal/mole into (4.23) – as previously obtained for an ionophore in a bilayer interior of $\epsilon_{hc} \approx 6$ – gives flip-flop times in the normally measured range 10^4–10^5 s (i.e. hours–days). The possible effects of proteins in enhancing flip-flop in real membranes will be discussed in Section V.

The slow rates for both flip-flop and exchange have important implications for membrane structure: as already mentioned they highlight the necessity for looking at the whole system rather than one part of it in considerations of the stability and equilibrium states of membranes. Thus membrane structures may require a long time to relax to equilibrium following an external mechanical perturbation or a chemical change in their environment (e.g. lipid or protein insertion). Such stimuli may cause membranes to respond via nonequilibrium relaxation processes such as induced flip-flop, phase separations, and non-equilibrium elastic shape changes. A consideration of the equilibrium thermodynamic state of bilayers and membranes may therefore not be relevant to certain aspects of their behaviour since such an analysis presupposes that different membrane aggregates have time to come to equilibrium through the exchange of lipids via the aqueous phase.

Bearing these qualifications in mind we shall now review the *equilibrium* properties of bilayer-forming lipids.

G. *Bilayer-forming lipids and bilayer shapes*

As already mentioned the geometric packing properties of different lipids may be conveniently expressed in terms of the dimensionless 'critical packing parameter', $v/a_0 l_c$, characteristic for each lipid, the value of which determines the type of aggregate formed. Fig. 4.2 lists the packing characteristics of some common biological lipids. If $v/a_0 l_c < \frac{1}{2}$ such lipids normally form micelles and are entropically and energetically unlikely to form bilayers. If $v/a_0 l_c > 1$ such lipids cannot even pack into bilayers since their head-group area is too small; instead, they form inverted micellar structures or precipitate out of solution (e.g. cholesterol). It is those lipids for which $\frac{1}{2} < v/a_0 l_c < 1$ that will be our main concern since these self-assemble into bilayer-type structures. But what determines the overall shapes of the bilayers? First, discrete disc-like bilayers are never observed when only one type

of lipid is present. The reason for this arises from the highly curved hemi-cylindrical rim of a (hypothetical) planar bilayer disc which, since $v/a_0 l_c > \frac{1}{2}$, would contain lipids of surface area $a > a_0$. Since the free energy per rim molecule would then be higher than that of molecules in the rest of the bilayer, the mean free energy per molecule in such an aggregate will be

$$\mu_N^0 = \mu_\infty^0 + \alpha kT/N^{\frac{1}{2}}; \tag{4.24}$$

this follows since for a disc of radius R the number of molecules N in the disc is proportional to R^2 while the number in the rim is proportional to R, and hence to $N^{\frac{1}{2}}$. From the discussion in Section III(D) we see that such a dependence implies that infinite bilayers are expected to form spontaneously from monomers at the CMC. Thus single component lipid bilayers with exposed edges should not exist.

Under certain conditions it becomes more favourable for closed spherical bilayers (vesicles) to form rather than infinite planar bilayers. This arises since in a closed bilayer the energetically unfavourable rim regions are eliminated at a finite, rather than infinite, aggregation number, which is entropically favoured. Thus so long as the curved bilayer lipids can maintain their areas at their optimal value vesicles should be the preferred structures. What then determines the radii of vesicles? For spherical vesicles, and indeed for all curved bilayers, the head-group area a and hence the energy μ_N^0 will depend on vesicle size. However, for bilayers or vesicles with radius of curvature greater than a certain critical radius R_c the lipids on both the inner and outer monolayers can pack with their surface areas at the optimal value a_0. Thus we have

$$\text{for } R > R_c \quad (l < l_c): \quad \mu_N^0 = 2\gamma a_0. \tag{4.25}$$

But for vesicles of radius less than R_c, since the chain length l cannot exceed l_c, packing restrictions now force the surface area a in the outer monolayer to increase above a_0 in such a way that the mean free energy per molecule, given by equation (4.16), may be expressed as (Israelachvili *et al.* 1977)

$$\begin{aligned} R < R_c \quad (l = l_c): \quad \mu_N^0 &= 2\gamma a_0 + \frac{4\pi l_c^2 \gamma}{N}[1 - R/R_c]^2 \\ &\approx 2\gamma a_0 + \frac{\pi l_c^2 \gamma}{M^3}(\Delta N)^2, \end{aligned} \tag{4.26}$$

where the *critical packing radius* is given by

$$R_c = l_c[3 + \sqrt{3(4v/a_0 l_c - 1)}]/6[1 - v/a_0 l_c] \approx l_c/(1 - v/a_0 l_c) \quad (4.27)$$

and where the aggregation number is

$$N \approx 4\pi[R^2 + (R-t)^2]/a_0, \quad (4.28)$$

t being the bilayer hydrocarbon thickness given by $t \approx 2v/a_0$.

Incorporating the above equations into (3.2) shows that the mean radius of a population of spherical vesicles is close to their geometric packing radius R_c and that the distribution is Gaussian with standard deviation in the aggregation number (substituting (4.26) into (3.12)–(3.14)):

$$\frac{\sigma_M}{M} = \sqrt{(kT/2\pi l_c^2 \gamma)} \approx 0{\cdot}05. \quad (4.29)$$

Alternatively, the standard deviation in the radius is

$$\frac{\sigma_R}{R_c} = \sqrt{(kT/8\pi l_c^2 \gamma)} \approx 0{\cdot}03. \quad (4.30)$$

Thus the distribution of vesicles may be expected to be fairly monodisperse, as observed (Brunner, Skrabal & Hauser, 1976).

The above equations show that larger vesicles ($R > R_c$) are thermodynamically unfavoured not because of any packing constraints (higher μ_N^0) but simply because the system would have lower entropy. Smaller vesicles, however, are unfavoured because of the higher energy associated with their increased surface area above a_0 in the outer monolayer (and, for very small vesicles, possible additional forces within the highly-curved inner monolayer – Mitchell & Ninham, 1980).

As an example we may apply the above equations to the much studied egg lecithin vesicles (for full details see Israelachvili *et al.* 1976, 1977) for which $a_0 \approx 71{\cdot}7$ Å^2, $v \approx 1063$ Å^3 and $l_c \approx 17{\cdot}5$ Å (i.e. $v/a_0 l_c \approx 0{\cdot}85$). Equations (4.27)–(4.28) then yield: $R_c \approx 108$ Å, $N \approx 3000$, $t \approx 30$ Å and an outside/inside ratio of $R_c^2/(R_c - t)^2 \approx 1{\cdot}9$. These are strictly geometric parameters; the full thermodynamic treatment shows that, depending on the lipid concentration, the vesicle population peaks at $R \approx 100$–105 Å, $\bar{N} \approx 2600$, and the outside/inside ratio is $\approx 2{\cdot}0$, i.e. not very different from that obtained from purely geometric considerations. For lysolecithin, using the same values for

a_0 and l_c as for lecithin, but with half the chain volume, leads to $v/a_0 l_c = 0{\cdot}42$. For such a packing parameter the theory predicts (Israelachvili *et al.* 1976, Fig. 8; Carnie *et al.* 1979) that lysolecithin should form small globular (non-spherical) micelles of aggregation number $\sim$ 186. This compares very well with the experimental value of 181, which shows that for lecithins the head-group area is dominated by head-group interactions. It is also interesting that the lecithin head-group conformation is the same in bilayers and micelles (Hauser *et al.* 1980*b*).

The above analysis so far provides a satisfactory quantitative account of such vesicle properties as their size, aggregation number, inside/outside ratio and polydispersity. It also leads to the following qualitative, and normally observed, effects (see also Fig. 4.2).

(i) Lipids with smaller head-group areas (high $v/a_0 l_c$) should form large vesicles, less curved bilayers, or inverted micellar phases. For anionic lipids this can be brought about by increasing the salt concentration, particularly Ca^{2+}, or lowering the pH. This also has the effect of straightening (condensing) the chains and reducing their fluidity.

(ii) Increased unsaturation, particularly of *cis* double bonds, reduces l_c and thus increases $v/a_0 l_c$. This leads to larger vesicles, and in general to more inverted-cone type lipids.

(iii) Increasing the temperature increases the hydrocarbon chain motion, involving *trans-gauche* isomerization, and thereby reduces their limiting length l_c. This again leads to an increased $v/a_0 l_c$. The above three effects of head-group size, ionic strength, unxaturation and temperature are illustrated in Fig. 4.2.

While the above treatment offers a rough and ready recipe for analysing the packing and thermodynamic properties of lipid vesicles it is nevertheless incomplete. The only restriction so far considered is that the chains cannot extend away from the head-group further than a certain distance l_c. This leads to an increased energy only for radii of curvature below R_c, but for $R > R_c$ there is no curvature dependence at all (cf. equation (4.25)) because of two simplifying assumptions that require further consideration:

(i) It is assumed that hydrocarbon chains are entirely fluid and do not oppose any distortion (until they become forced to extend beyond l_c). In other words, it is assumed that there is no curvature dependence of μ_N^0 due to the non-fluidity of the chains.

(ii) The attractive and repulsive interfacial forces which determine

a_0 and μ_N^0 (equations (4.14) and (4.15)) are both assumed to act in the same plane, at the hydrocarbon–water interface, at which the surface area per molecule has been defined. This is likely to be true for the attractive interfacial tension force but not for the head-group and chain repulsive forces which are likely to be centred respectively above and below this interface (Fig. 4.1). Again, this effect could lead to a curvature dependence of μ_N^0.

We must now relax the above assumptions and examine the curvature elasticity of bilayer membranes.

H. *Curvature elasticity of bilayers*

As mentioned above, deformation of a bilayer membrane involves changes in molecular conformations in both the hydrocarbon chain and polar head regions. As discussed in Section II, the picture at the molecular level is quite complex and not fully understood, and further complications arise in multicomponent systems where local membrane bending can also lead to a lateral and vertical rearrangement of molecules between differently curved regions and across the two sides of the membrane (we have as yet not considered multicomponent membranes, this being reserved for Section V).The bending properties of membranes have traditionally been described by one or other of the *continuum* (or *macroscopic*) theories, wherein a bilayer or membrane is considered to act as a classical continuous elastic sheet or as an elastic lamellar liquid crystal. The molecular structure of the membrane does not directly enter into the classical elasticity equations. For surfaces which are much larger than molecular dimensions, and for small deformations, such an approach may be expected to be valid and successful. Indeed, this approach has allowed for certain macroscopic properties of bilayers and membranes, such as their overall shape, to be quantitatively predicted in terms of variously-assigned elastic moduli. However, such entropy-dependent factors as inter-membrane lipid exchange and structural aspects at the molecular level cannot be handled by elasticity theories, which are anyway not primarily concerned with the mechanism of self-assembly since the structures analysed are not derived but assumed to exist from the outset.

Within the continuum approach, membrane elasticity has been studied in considerable detail by Evans and coworkers. Their work has been recently described in review articles (Evans & Hochmuth, 1978; Evans & Skalak, 1979). It covers the whole area from a general and

rigorous elastic description of a continuous sheet to many convincing applications, particularly to red blood cell shapes.

A simpler, more intuitive approach has been introduced by Helfrich (1973), and more recently extended by Petrov, Seleznev & Derzhanski (1978). It is based on a general quadratic form for the energy change associated with curvature of the membrane surface. Helfrich neglects the energy associated with shear deformations. In the spirit of the fluid mosaic model, such an assumption is justified for bilayers with fluid lipid chains, where lateral diffusion of molecules is rapid, the two monolayers may be considered free to slide over each other, and therefore no significant shear resistance could be expected. It is however less tenable for frozen or rigid bilayers, or for the membrane of the erythrocyte with its underlying network of spectrin fibres. Nevertheless, erythrocyte shapes calculated by Deuling & Helfrich (1976) are in good agreement with experiments.

In considering the origins of the various elastic moduli used in the continuum theories, two limiting cases are recognized:

(i) There is no lipid exchange between the two sides of the bilayer (i.e. the two sides are 'coupled');

(ii) There is a free exchange of lipids between the two sides of the bilayer ('uncoupled').

It is not difficult to imagine an intermediate case (which indeed is most likely to be encountered) and which is seldom, if ever, discussed in the literature. Clearly, the elastic moduli in case (ii) above will be much smaller than in case (i). Nevertheless, a bending in case (ii) involves a change in molecular conformation, and the elasticity will not be zero as assumed by Evans & Skalak (1979). In order to obtain a better picture of the elastic deformation at the molecular level it would be necessary to consider separately the deformation of lipid chains and the deformation of polar heads caused by bilayer bending. While the elastic energy associated with chain deformation could be computed relatively accurately using the methods described in Section II, the polar head contribution remains unknown. A reliable microscopic picture of the elastic deformation in bilayer membranes is therefore still not available.

I. *Relation between bilayer elasticity and lipid packing*

In previous sections we have discussed the formation of lipid aggregates on the basis of a simplified form for the free energy, which included contributions resulting from the removal of lipid tails from the aqueous environment, surface tension, head-group repulsion and elementary packing requirements. These represent the dominant contributions and in many cases are sufficient for understanding aggregation processes. In other cases, however, the free energy of alternative forms of aggregates

is relatively similar, and theoretical considerations must be broadened to include a further contribution which arises when bilayers beome curved.

Much insight into the effect of molecular properties on bilayer bending and vesicle size can be obtained by the following simple modification of the molecular theory discussed so far: let us now assume that the repulsive force effective acts at some distance above (or below) the interface where the attractive forces are acting. It is easy to show that if the centre of the repulsion is located at distance D away from the hydrocarbon–water interface the free energy of (4.14) becomes replaced by (Israelachvili *et al.* 1976, 1977):

$$\mu_N^0 = \gamma a + \frac{C}{a(1+D/R)}, \tag{4.31}$$

where R is the local radius of curvature of the surface. If the head-group repulsion dominates the repulsive force D will be positive, whereas if the non-fluidity of the chains dominates D will be negative (see Fig. 4.1). The leading contribution in other more complicated models for the effect of curvature elasticity always has the form of (4.31).

The free energy per molecule in a vesicle may be expressed in terms of the inner and outer radii and head-group areas. Minimization of the free energy then determines the vesicle parameters. Representative results are given by Mitchell & Ninham (1980). Two levels of approximation are natural to the problem.

(1) For low bilayer curvature, linearization is acceptable, and the area per molecule remains near its unperturbed value, α_0.

The free energy per molecule in a vesicle can now be shown to be:

$$\mu_N^0 = 2\gamma a_0 - 4\pi\gamma Dt/N \qquad \text{for } R > R_c \tag{4.32}$$

$$\mu_N^0 = 2\gamma a_0 + \frac{4\pi l_c^2 \gamma}{N}(1 - R/R_c)^2 - \frac{4\pi Dt\gamma}{N} \qquad \text{for } R < R_c, \tag{4.33}$$

which reduces to (4.25)–(4.26) when $D = 0$. A finite value for D now affects the variation of μ_N^0 with N at all values of N (and R), and the picture changes depending on the sign and magnitude of D. This will now be considered.

(i) If the repulsion between the head-groups dominates the net repulsive force, D will be positive, and of magnitude that will depend on the head-group length, size and conformation, and, for ionic head-

groups, on the ionic strength. Typical values will be of the order of a few Ångstroms. Thus a finite D head-group repulsion results in a small additional negative term to the free energy which becomes more negative as N falls and therefore favours even smaller vesicles. The net effect is that the vesicle distribution will now peak at a radius R_{peak} less than R_c

$$R_{\text{peak}} \approx R_c[1 - Dt/l_c^2], \tag{4.34}$$

and that the standard deviation (polydispersity) will increase above that given by (4.29) and (4.30). From (4.34) we see that, since $t \approx 2l_c$, vesicles with large repulsive head-groups and short chains should be very small, and that once the hydrocarbon chain length falls below $\sim 2D$ no stable vesicles should form; instead, micelles should be formed, probably rod-shaped, as occurs for short-chained (C_{10}, C_{12}, C_{14}) lecithins.

Equations (4.32)–(4.33) show that with $D > 0$ the bending of a bilayer now becomes energetically favourable right down to the critical packing radius. Helfrich (1974) has postulated the existence of a 'spontaneous curvature', equivalent to the $(1 - R/R_c)^2$ term in equation (4.33), in bilayer and vesicle shape considerations though its origin was not explained. Indeed it is interesting to note that for any value of D bilayers oppose bending *beyond* R_c by increasing their energy *per unit area* by

$$\begin{aligned}\frac{2\mu_N^0}{a_0} &\approx \frac{4\gamma a_0}{a_0} + \frac{8\pi l_c^2\gamma}{Na_0}(1 - R/R_c)^2 \\ &\approx \text{Constant} + \frac{2l_c^2\gamma R^2}{[R^2 + (R-t)^2]}\left(\frac{1}{R} - \frac{1}{R_c}\right)^2 \\ &\approx \frac{1}{2}k_c\left(\frac{1}{R} - \frac{1}{R_c}\right)^2 \quad \text{for} \quad R < R_c.\end{aligned} \tag{4.35}$$

Such a curvature dependence has the same form as that for a curved elastic membrane (Helfrich, 1974) of elastic curvature modulus k_c, given by

$$k_c \approx 2l_c^2\gamma. \tag{4.36}$$

Thus for $l_c \approx 17{\cdot}5$ Å: $k_c \approx 3 \times 10^{-12}$ erg – a value that should roughly apply to all highly-curved bilayers and vesicles in the fluid state. The above value is similar to $k_c \approx 2{\cdot}6 \times 10^{-12}$ erg estimated by Helfrich (1974) by a completely different method.

(ii) If the repulsion between the hydrocarboten chains dominas, D will be negative, and the picture changes completely. Thus even for

large vesicles ($R > R_c$), where packing constraints are not important, equation (4.32) becomes

$$\mu_N^0 = 2\gamma a_0 + 4\pi Dt\gamma/N = \mu_\infty^0 + \alpha kT/N, \tag{4.37}$$

where

$$\alpha = 4\pi Dt\gamma/kT \tag{4.38}$$

(cf. equation (3.5)).

The first important consequence of a negative D is that it leads to equations that are formally indistinguishable from a bilayer (or planar sheet) which bends elastically at all radii! To show this we note that the free energy *per unit area* of vesicle bilayer now becomes (putting $N \approx 8\pi R^2/a_0$)

$$\begin{aligned} \frac{2\mu_N^0}{a_0} &= \text{Constant} + Dt\gamma \left(\frac{1}{R}\right)^2 \\ &= \text{Constant} + \frac{1}{2} k_c' \left(\frac{1}{R}\right)^2 \end{aligned} \tag{4.39}$$

which is equivalent to (4.35) with $R_c = \infty$, i.e. the bilayer has a purely elastic bending energy that opposes bending from the planar state, with a curvature or bending elastic modulus k_c now given by

$$k_c' = 2Dt\gamma = \alpha kT/2\pi. \tag{4.40}$$

This elasticity is due to the compression of the fluid chains at all R, and should be distinguished from that occurring only for highly-curved bilayers at $R < R_c$ where the elastic modulus (4.36) is higher since the chains are now fully extended.

A further important consequence of a negative D is that since $\mu_N^0 = \mu_\infty^0 + \alpha kT/N$ the mean aggregation number $\bar{N}$ of vesicles composed of such elastic bilayers is now given by (3.10), viz.

$$\bar{N} \approx 2\sqrt{Se^{\alpha}} = 2\sqrt{Se^{2\pi k_c'/kT}}, \tag{4.41}$$

where S is the lipid concentration (in Mol/55.5). Thus for a typical lipid concentration of 10^{-3} M and below we see that only if $k_c' \ll 10^{-13}$ erg will vesicle populations be small and monodisperse, with radii $R \approx R_c$. But if $k_c' \gg 10^{-13}$ erg vesicles will be large and polydisperse, and their size will increase with the total lipid concentration.† Such

† A simple illustrative example which shows the competitive effects of entropy, which favours smaller vesicles, and elastic bending energy, which favours larger vesicles, can be given by considering a large vesicle of aggregation number N whose bending elastic energy is, according to (4.39): $\frac{1}{2} k_c'(1/R)^2 . 4\pi R^2 = 2\pi k_c$ per vesicle. If this large vesicle breaks up into n smaller vesicles then the change in elastic energy is $+(n-1)2\pi k_c'$ while the gain in translational entropy is $(\frac{3}{2})\ (n-1)kT$. Thus if $2\pi k_c' > (3/2)kT$, or if $k_c' > 10^{-14}$ erg, this process becomes unfavourable, whereas if $k_c' < 10^{-14}$ erg smaller vesicles are favoured. This example is not rigorous but serves to illustrate the importance of entropy. The rigorous treatment was given above.

lipids may be identified with the 'inverted cone' lipids of Fig. 4.2. For example, lipid vesicles prepared in alcohol–water mixtures appear to fall into this category (Kremer *et al.* 1977); indeed, addition of alcohols to surfactant–water mixtures generally increases their packing parameter, $v/a_0 l_c$, and thereby promotes lamellar phase formation (Wennerström, 1979; Wennerström & Lindman, 1979, p. 24).

(2) For very small vesicles (of high curvature) the inner hydrocarbon monolayer becomes unrealistically thin, which is clearly a state of unfavourable packing for lipid chains. A new weak packing criterion now has to be introduced in order to obtain sensible results: there will be a minimal chain length below which the chain free energy increases such that any shorter configuration is ruled out. In addition, the head-group areas are far from optimal, and non-linear solutions for the vesicle free energy are necessary. The geometrical parameters of a vesicle are then obtained only after a tedious numerical minimization of the free energy (Mitchell & Ninham, 1980). However, the results obtained with the linearized and non-linear theories are qualitatively similar, and substantial predictions of the non-linear theory still depend only on the parameter $v/a_0 l_c$ and are independent of the detailed form of the forces. As $v/a_0 l_c$ decreases from unity, the optimal vesicle size decreases as in the linear theory until about $v/a_0 l_c \sim 0{\cdot}7$ (radius $\sim$ 50 Å) below which the effect of internal packing constraints become important.

At present there is some experimental controversy about whether vesicle suspensions are ultimately stable or not. The present treatment cannot resolve this issue since, depending on the attractive forces between bilayers and the sign and magnitude of D – difficult parameters to compute – either the vesicle or the bilayer (multi-layer) state can emerge as the stable one (see below). Our main concern has been to determine the physical parameters and thermodynamic criteria for stability. For large ionic head-groups in low salt and fluid chains ($T > T_t$) we may well expect D to be positive, and vesicle suspensions to be stable. For lipids with small head-group areas, or in the frozen state ($T < T_t$) where the chains are rigid and fully extended, k_c is large, and only planar bilayers or multilayers will form. Indeed when small dipalmitoylphosphatidylcholine vesicles are cooled below T_t they become polygonal – possessing about 45 planar crystalline bilayer facets per vesicle (Blaurock & Gamble, 1979).

J. *Multilayers and liposomes*

Before we leave the subject of isolated lipid structures it is worth considering, if only crudely, the effect of attractive forces between membranes in determining their overall stability. These can lead to the formation of ordered multilayered structures whose importance and occurrence as cellular organelles are no less than those of isolated vesicles and membranes (e.g. photoreceptor and thylakoid membranes, cell junctions, myelin). The effect of membrane interactions on membrane structure is described in the following section; here we shall only consider the strength of interaction needed to stabilize such lamellae.

At the end of the previous section it was shown that if $\Delta\mu$ is the free energy *per molecule* in vesicles (or micelles) relative to that in multilayers, a vesicle-to-multilayer transition will occur at a critical concentration of $X = M \exp(-M\Delta\mu/kT)$ above which only multilayers will be formed, where M is the vesicle or micelle aggregation number. Taking $M \approx 3000$ as a typical aggregation number for vesicles of 200 Å diameter, we see that if $\Delta\mu > 0{\cdot}01kT$ multilayers will form already at the CMC of $\sim 10^{-10}$ mole/litre, while if $\Delta\mu = 0{\cdot}006kT$ the transition will occur at a higher concentration of $\sim 10^{-3}$ mole/litre ($\sim$ 1 mg/ml). For molecules of surface area ~ 60 Å^2 these values correspond to surface interaction energies in the range 0·04–0·07 erg/cm^2. For larger vesicles correspondingly smaller energies are needed to initiate the transition; these being given roughly by $(200/M)$ erg/cm^2.

If curvature effects are ignored the main contribution to $\Delta\mu$ arises from the favourable interaction energy between multilamellar bilayers in which the equilibrium bilayer separation occurs at a potential energy minimum, determined by the balance of the attractive and repulsive forces. Theoretical estimates of this energy for ionized membrane surfaces range from 0 in low salt, to 10^{-4}–10^{-3} erg/cm^2 in $\sim$ 0·145 M monovalent electrolyte solutions, to $\sim$ 0·1 erg/cm^2 for uncharged (or Ca^{2+} neutralized) surfaces in contact (Parsegian & Gingell, 1972). Thus ionized membranes in low salt should not form stable multilayers in excess water. This agrees with the qualitative observations of Papahadjopoulos & Miller (1967) and Papahadjopoulos *et al.* (1976) that multilayers composed of anionic lipids disperse into large vesicles in 0·145 M KCl or NaCl or low salt after Ca^{2+} is removed or the pH increased – both of which increase the electrostatic repulsive forces. Actual

measurements of intermembrane surface free energies, as opposed to adhesion forces, are not readily available. The one direct measurement of the forces between lecithin bilayers (Le Neveu *et al.* 1977) suggests a value of $\sim 10^{-2}$ erg/cm^2 for the depth of the interaction energy minimum of lecithin bilayers at their equilibrium separation of ~ 27 Å (which should make a dilute suspension of vesicles just stable), while Buxbaum (1980) obtained a value of $\sim 10^{-4}$–5×10^{-3} erg/cm^2 for red cell membranes. It is interesting that these values are close to those needed to induce stacking of membranes in dilute dispersions, and the cell no doubt makes use of this fine balance in its control of stacking–unstacking processes.

At high lipid concentrations ($> 10\%$) repulsive forces alone can force lipids to form into multilayers, an effect that can also be brought about by centrifugation.

V. Structure of multicomponent membranes

So far we have primarily discussed single-component systems, e.g. pure lipid bilayers, for which it has been possible to obtain qualitative and sometimes quantitative predictions using the concepts outlined in Sections III–IV. Now we turn our attention to membrane systems of more than one component. It is beyond our scope to obtain a quantitative understanding of the structure of multicomponent lipid–protein membranes in terms of basic principles, for real biological membranes or even reconstituted model membranes are very complex: they commonly contain 50 or more different proteins and a host of phospholipids and glycolipids with various head-groups, numbers of chains, chain lengths and degrees of unsaturation, as well as steroids, pigments and other amphiphilic molecules. Yet in spite of these complexities there are many aspects of membrane structure that may be qualitatively understood in terms of the principles we have discussed, e.g. such phenomena as lateral heterogeneity, phase separation and clustering, boundary lipids, protein aggregation, membrane asymmetry and membrane shape changes (Cullis & de Kruijff, 1979; Israelachvili, 1978).

A membrane is a small thermodynamic system (Hill, 1963), and the thermodynamic ideas developed earlier can be applied to it. For example, the question of whether or not certain membrane components will aggregate or remain dispersed in the membrane is decided by a balance between energy and entropy in the same way as such a balance

determines the assembly of lipids in water into various aggregates (Section III). In a *multicomponent* membrane the *entropy of mixing* enters as an additional factor which always favours randomization and dispersal of the molecules. The forces that hold lipids and proteins together in membranes, and give membranes their characteristic fluid-like properties of flexibility and permeability, are not strong covalent bonds but arise from weaker hydrophobic, hydrogen-bonding and electrostatic interactions. When these are not too selective, the entropy of mixing wins out and the membrane components remain randomly dispersed. Often, however, two molecules or types of molecule have a particularly strong attraction so that they are more likely to be found together in clusters or in a separate phase. In such cases we talk of a 'specific interaction', although it should be remembered that the distinction between 'specific' and 'nonspecific' is only one of degree.

The strong interactions which can lead to association (or segregation) of particular components in the membrane include steric hindrances and packing limitations. For example, rigid molecules such as steroids and proteins will in general not be able to pack together. However, the acyl-chained lipids of most natural membranes are in the fluid state at physiological temperatures and can easily accommodate each other as well as other molecules in a bilayer configuration. The difference in packing properties between rigid and flexible molecules is an important one which will often reappear in this section. Indeed, as far as membrane *structure* is concerned, the distinction between lipids and proteins is artificial: a classification into rigid molecules (e.g. steroids, proteins) and flexible molecules (e.g. lipids above T_t, undfolded proteins) is much more useful.

A feature of lipid molecules which has particularly interesting ramifications for membrane structure is the interdependence of the free energy and geometry of the molecule. As discussed in Section II the free energy of phospholipid molecules in the fluid state depends on the average molecular shape, i.e. on the available chain conformations which are determined by the interaction of a given chain with its neighbours. However, if the shape is very restricted, for example allowing only a few conformations or only high-energy conformations, the free energy increases dramatically. Thus chains cannot be forced by a neighbouring molecule to extend beyond a certain critical length (close to the fully-extended length) since such extensions become entropically prohibitive.

This feature was discussed in Section IV, where it was shown that a simple model incorporating this 'packing limit' gives quantitative predictions about the self-assembly of lipid molecules in aqueous media.

In this final section we review the structure and function of multi-component membranes and consider how far some of these may be understood in terms of packing constraints and other interactions. In particular, we should not succumb too readily to the practice of invoking hypothetical enzymes to explain a variety of membrane-associated phenomena (e.g. flip-flop); many of them may be explicable by simple thermodynamic considerations of membrane structure.

A. *Mixed lipid bilayers*

Lipids in the fluid state generally mix homogeneously in bilayers (e.g. Shimshick & McConnell, 1973; Mabrey & Sturtevant, 1976) since their chains can distort and mutually accommodate each other (Fig. 5.1*a*). Only rarely is there any immiscibility between fluid-phase lipid (e.g. Wu & McConnell, 1975), and then only if there is a significant difference in the head-group areas or chain lengths of the different lipids so that they are unable to pack together easily in a bilayer even in the fluid. state (Fig. 5.1*b*). Segregation between anionic and neutral lipids can be induced by cation binding, e.g. Ca^{2+} (Ohnishi & Ito, 1973; van Dijck *et al.* 1978) and polylysine (Hartmann, Galla & Sackmann, 1977). Such bonding is often stochiometric, e.g. one Ca^{2+} per two anionic sites, and leads to an attractive electrostatic association of anionic head-groups.

In a mixed-lipid bilayer, if the chains of one or more of the lipids are in the fluid state the packing criteria of Section IV can be applied to give quantitative predictions, for example, the size and asymmetric lipid distribution of mixed lipid vesicles (Carnie *et al.* 1979). Thus addition of lysolecithin to lecithin vesicles results in smaller vesicles since such a mixture of lipids can pack into more highly curved bilayers (cf. Fig. 4.2). Further, entropy favours smaller vesicles, and since this can be brought about if more lysolecithin becomes incorporated into the outer vesicle monolayer, the vesicles are asymmetric. In general all mixed lipid vesicles will be asymmetric if the packing of the molecules is different – the ultimate asymmetry being determined by the unfavourable entropy of demixing between the inner and outer monolayers. On the other hand, phosphatidylethanolamine and cholesterol both have the shape of an inverted truncated cone (Fig. 4.2) and these

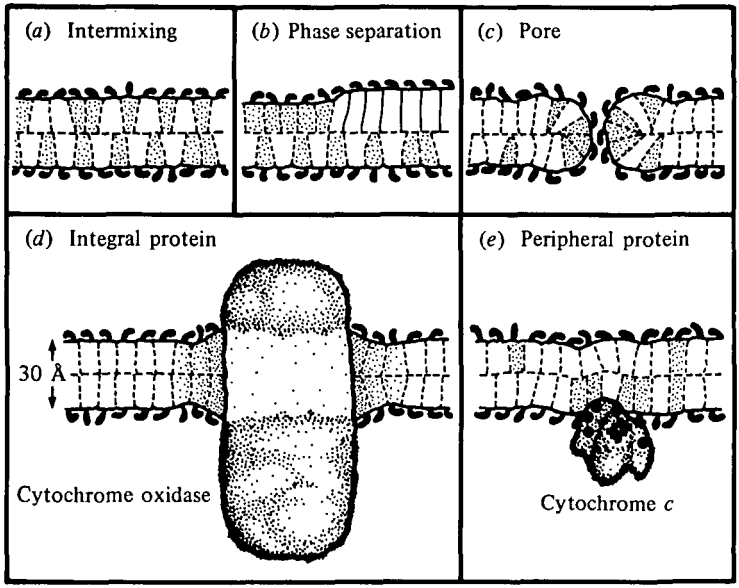

Fig. 5.1. Scaled drawings of mean packing conformations of mixed lipid and lipid–protein membranes. In each case the unperturbed bilayer hydrocarbon thickness is 30 Å. (*a*) Mixed lecithins of head-group area ~ 70 Å^2 but of different chain lengths, in the fluid state. (*b*) Solid-liquid phase separation in upper monolayer of a bilayer. Note that packing stresses must occur at the boundary. (*c*) Mixture of lecithin and lysolecithin where a transient local clustering of lysolecithin can produce a pore or channel. (*d*) Cytochrome oxidase in a lecithin bilayer. Note the perturbations at the protein boundary which may preferentially draw in certain lipids. Dimensions for cytochrome oxidase taken from Henderson *et al.* (1977); dimensions for lecithin are as discussed in Section IV. The detailed shape of cytochrome oxidase is still unknown. (*e*) Cytochrome *c* preferentially associating with charged lipids in a mixed-lipid bilayer via basic lysine residues of the protein surface. Dimensions for cytochrome *c* and positions of the haem and the five invariable lysine residues taken from Dickerson *et al.* (1971).

when mixed with lecithin increase the vesicle size – the asymmetry now going in the opposite direction. Only a limited amount of cholesterol and phosphatidylethanolamine can be incorporated into bilayers before the bilayer structure is desctroyed (Cullis & de Kurijff, 1978; Israelachvili & Mitchell, 1975) at the point where the fluid lipids can no longer accommodate these inverted-cone lipids. Indeed, natural biological membranes never contain high amounts of both cholesterol and phosphatidylethanolamine, e.g. of these two lipids the myelin membrane contains only cholesterol whereas the inner mitochondrial membrane has only phosphatidylethanolamine, while the erythrocyte membrane contains both but on opposite sides. Further, in three-

component bilayers cholesterol and phosphatidylethanolamine associate with lecithin rather than with each other due to their incompatible packing properties (van Dijck *et al.* 1976). However, cholesterol and lysolecithin – neither of which can form a stable bilayer by itself – can combine in certain proportions to form bilayers (Kitagawa, Inoue & Nojima, 1976).

Small amounts of lysolecithin can be incorporated into bilayers before they break up into small vesicles or micelles, but before this critical concentration is reached the lysolecithin molecules can themselves associate to form transient pores as indicated in Fig. 5.1*c*. In accordance with this idea, it has been found that addition of lysolecithin to lecithin vesicles or liposomes increases their permeability to ions and small molecules; however, cholesterol counteracts this effect (Kitagawa *et al.* 1976; Lee & Chan, 1977; Ralston *et al.* 1980).

If the packing equilibrium between the two sides of a bilayer is disturbed, for example by changing the optimal head-group area or incorporating new lipids into one side of the bilayer, the rate of flip-flop may be enhanced to restore the lipid distribution to one which satisfies their packing requirements (de Kruijff & Baken, 1978; Cullis & de Kruijff, 1979; Carnie *et al.* 1979). Such effects also lead to membrane shape changes as discussed later.

Phase separation into fluid and solid lipid domains is a characteristic of the chain freezing transition in mixed lipid systems. Such domains have been detected by electron microscopy (Hui & Parsons, 1975; Luna & McConnell, 1978), spin-label partitioning (Shimshick & McConnell, 1973), differential scanning calorimetry (Mabrey & Sturtevant, 1976), and in monolayer studies (Taylor *et al.* 1973). These investigations have furnished phase diagrams of binary lipid mixtures. A phase separation on freezing is a general feature of lipid mixtures, but the detailed form of the phase diagram depends on the interaction between the constituent molecules. Lee (1977) has reviewed the possible forms of the phase diagrams depending on the miscibility or immiscibility of the components and various deviations from ideal mixing. Papahadjopoulos (1977) has discussed the effect of Ca^{2+} binding on increasing the freezing temperature of anionic lipids, e.g. phosphatidylserine, by 30 °C or more – an effect that at a given temperature leads to phase separation due to a shift in T_t. When frozen and fluid lipids phase separate they usually do so into domains containing different mixtures of each species.

A further type of phase separation, or immiscibility, exists in which some molecules aggregate exclusively into a finite domain. If such a domain contains N molecules there will always be a contribution to the free energy per molecule μ_N^0 from interactions with the surrounding bilayer at the domain boundary. This contribution to the free energy μ_N^0 is proportional to $N^{-\frac{1}{2}}$, so that for the case of ideal mixing we may write

$$\mu_N^0 = \mu_\infty^0 + \alpha kT/N^{\frac{1}{2}}.$$

In Section III(D) we saw that such a dependence leads to a CMC at a mole fraction given by CMC $\approx e^{-\alpha}$ at which point a lateral phase separation will occur. Such an analysis should be applicable to large aggregates in membranes, e.g. capping.

Before we turn our attention from lipid–lipid to lipid–protein interactions it is well to return to a consideration of steroids in general and cholesterol in particular. Cholesterol is considered as a lipid, but it is a comparatively rigid molecule and lacks the accommodating ability of acyl-chained lipids. In particular cholesterol does not form bilayers, and its shape can be modelled as an inverted cone (Fig. 4.2). When cholesterol is mixed with bilayer- and vesicle-forming lipids the different molecules cannot *mutually* accommodate each other as when both are fluid. In this case, only the hydrocarbon chains of the fluid lipids distort and this gives rise to an apparent 'condensing effect' on the host lipid: a straightening of the hydrocarbon chains, thickening of the bilayer, and reduced fluidity. All these effects may be qualitatively understood in terms of the rigid packing shape of cholesterol (Israelachvili & Mitchell, 1975; Carnie *et al.* 1979) without invoking specific binding. Because of its rigid shape, cholesterol always partitions into the fluid phase when there is a phase separation (Kleeman & McConnell, 1976), and – as mentioned – in mixed lecithin–phosphatidylethanolamine bilayers cholesterol associates preferentially with the former. Even at high concentrations, cholesterol molecules are not found together, but remain separated by at least one fluid lipid molecule (Engelman & Rothman, 1972). The fluid lipid molecules in this case play a similar role to the boundary lipids around proteins. Indeed, because of their rigidity it is more fruitful to treat cholesterol and proteins together when invoking packing considerations for the arrangement of components in membranes.

B. *Proteins in membranes*

Compared to acyl-chained lipids, integral membrane proteins are structurally rigid, and when incorporated into lipid bilayers it is inevitable that they will affect the lipid structrue in their vicinity. A perturbed lipid structure near a protein molecule was first detected by spin-label experiments (Jost *et al.* 1973; Stier & Sackmann, 1973). It was called 'boundary lipid', 'annulus' or 'halo' by different research groups. Many different integral and peripheral proteins are now known to perturb to varying degrees up to four lipid layers, e.g. cytochrome *c* oxidase, cytochrome *c*, polylysine, ATPase, myelin, proteolipid, *E. coli* proteins, rhodopsin and thylakoid membrane proteins. These perturbations manifest themselves in the ordering, phase transition behaviour, and fluidity properties of the lipids (Chapman, Gómez-Fernández & Goni, 1979; Papahadjopoulos, 1977; Hesketh *et al.* 1976; Papahadjopoulos *et al.* 1975); they have functional consequences as will be discussed.

The interaction between lipid and protein molecules covers the full range from strong specific binding to weak non-specific interactions resulting from close approach of the molecules. Operationally, two types of lipid-protein interactions may be distinguished: those that lead to specifically-bound lipids, and weaker interactions that merely perturb the lipid environment without necessarily giving rise to any preferential association, or to only a weak statistical association. Strongly-bound lipids copurify with their proteins and will not be considered in the present discussion of boundary lipids ('boundary' in the sense of 'occurring at the boundary', not in the sense of 'bound').

With cytochrome *c* oxidase ESR and MNR results give complementary information on the rate of exchange between the boundary layer lipids and the bulk. In ESR experiments the two spectral components corresponding to the boundary lipid and the free lipid are clearly resolved, indicating that the exchange rate is slower than $10^{-8} - 10^{-7}$ s (Jost & Griffith, 1980). On a much longer time scale, NMR measurements indicate a homogeneous lipid environment (Seelig & Seelig, 1978). The exchange rate between the boundary lipid and the bulk is thus faster than 10^{-4} s. Anionic, neutral and cationic lipids form a boundary around this protein to different degrees. Negatively-charged lipids are preferentially aggregated except in high salt where the anionic lipids is abolished (Brotherus *et al.* 1980). This clearly indicates that both hydrophobic and electrostatic interactions are

involved in this boundary system – an example of a more general conclusion recognized by Kimelberg & Papahadjopoulos, as early as 1971.

A further interesting phenomenon has been observed by Boggs *et al.* (1977) arising from the preferential association of lipophilin with anionic lipids. This depletes the rest of the membrane of anionic lipids and so changes *its* properties, e.g. T_t, in addition to the effect on the boundary lipids.

In the vicinity of integral proteins the lipid bilayer must distort in order to accommodate the irregular shape of a protein (Fig. 5.1 *d–e*). In addition to any possible electrostatic or hydrogen bonds, the general packing criteria are the same as for the self-assembly of lipid structures discussed earlier: the fluid hydrocarbon chains of acyl-chained lipids can deform whilst maintaining their surface area near the optimal value and at the same time not extending the chains beyond the maximum allowable length. In this way high-energy void regions in the membrane are eliminated (Israelachvili, 1977). Such packing accommodations around a protein will usually involve more than one boundary lipid layer and may also affect the lipids on the opposite side of the bilayer as illustrated in Fig. 5.1 *d–e*. Indeed, due to the co-operative nature of lipid chain interactions any perturbation of chain ordering cannot be restricted to only the first neighbours (Marčelja, 1976), although this calculation indicates that the perturbation does decay rapidly, typically within 2 or 3 lipid diameters away from the protein surface. We may expect that lipids with different head-groups or chain lengths will be affected to different extents (Boggs & Moscarello, 1978*b*). A particularly strong effect, corresponding to 3 or 4 concentric layers, involving about 140 lipids, has been reported in proteolipid apoprotein of myelin-lecithin recombinant membranes (Curatolo *et al.* 1977; Boggs *et al.* 1980).

Given the high protein/lipid ratio of natural membranes it is likely that no unperturbed lipid bilayer regions exist in real membranes.

Is it possible to understand more precisely the nature of the lipid chain perturbations introduced by protein molecules? Early spin-label experiments (Jost *et al.* 1973; Warren *et al.* 1975) indicated an *immobilized layer*, i.e. motionally restricted on the time scale of ESR measurements, the extent of which roughly corresponds to the number of lipid molecules, 30–50, in direct contact with the proteins. Later, differential scanning calorimetry experiments in lipid–protein bilayers showed a broadening of the chain-melting transition and a decrease in

transition enthalpy (Papahadjopoulos, 1977; Curatolo *et al.* 1977). This indicates a loss of co-operative chain–chain interaction, but does not provide details about the state of the boundary chains. Applications of deuteron magnetic resonance, which has the advantage of a non-perturbing molecular probe, has not been as informative as it was for pure lipid bilayers. With the addition of protein to a fluid lipid bilayer, the DMR order parameter usually decreases, particularly at the terminal methyl position (Kang *et al.* 1979). However, for some proteins, e.g. gramicidin A, the order parameters along the chain initially increase, and then decrease to low values at higher protein/lipid ratios (Rice & Oldfield, 1979). For some proteins the effect is very small; witness the slight decrease in order parameter along the whole lipid chain in proteolipid apoprotein–lecithin systems (Rice *et al.* 1979). But Raman scattering techniques applied to the same system (Curatolo *et al.* 1978) indicate that within boundary lipids the number of *trans* states is increased, suggesting an *increased* order. A similar conclusion has been reached by analysis of fluorescence anisotropy data, which measures the *local order* of lipid chains (Jähnig, 1979).

When results with different proteins are compared, one comes to the conclusion that the perturbation of lipid order is too specific to be described by a single picture. The seemingly contradictory results on some systems can be reconciled (Jähnig, 1979; Rice *et al.* 1979). For example, while the proteolipid apoprotein of myelin *increases* the local order of neighbouring lipid chains, the average orientation of boundary lipids is no longer along the bilayer normal (cf. Fig. 5.1*d*), and the usual DMR order parameter is therefore slightly *decreased.*

Peripheral proteins, too, can interact with bilayer lipids – mainly via electrostatic interactions – and hence affect their structure (cf. Fig. 5.1*e*). Thus the non-penetrating protein cytochrome *c* can bind to negatively-charged lipid head-groups and thereby induce them to phase separate or cluster under the protein, in similar fashion to the action of Ca^{2+} on anionic–neutral lipid mixtures (Birrell & Griffith, 1976).

Changes in the conformational state of proteins can alter the properties of their boundary lipids, as occurs when rhodopsin is illuminated (Favre *et al.* 1979). But it is perhaps well to point out that the effect of proteins on lipids is reciprocated: the proteins, too, are affected. While it is too early to say definitely whether lipid–protein interactions, packing stresses or lateral chain pressure induce conformational changes on proteins *in vivo* (they certainly affect activity as

discussed later) such interactions do affect the lateral organization and vertical displacement of proteins in membranes – e.g. their degree of penetration (Boggs & Moscarello, 1978*a*; Armond & Staehelin, 1979).

The less fluid a bilayer the less easily a protein may be expected to pack into it. For this reason cholesterol is rarely found in the boundary region of proteins (Boggs & Moscarello, 1978*a*; Warren *et al.* 1975). This is a further example of a simple packing mismatch, as was earlier discussed in more detail concerning phospholipid–cholesterol mixtures, and no specific interactions need be invoked. Thus addition of cholesterol can lead to an increased exposure of proteins (Borochov & Shinitzky, 1976) as can lowering the temperature (Armond & Staehelin, 1979) – both of which decrease the accommodating capacity of membranes.† It is also noteworthy that almost all the protein of the erythrocyte membrane is in the inner half which is the more fluid side of this membrane (Bretscher, 1973).

There is ample evidence that boundary lipids regulate the function of membrane proteins. A very informative review is available (Sandermann, 1978). The general conclusion which arises from many investigations is that in order to be active membrane proteins need their boundary lipid environment, and the level of activity increases with the fluidity of the surrounding lipid (Vik & Capaldi, 1977; Silvius & McElhaney, 1980). For example, in reconstitution experiments with cytochrome *c* oxidase the level of activity was found to be closely correlated with lipid fluidity and insensitive to the phospholipid headgroup (Vik & Capaldi, 1977). Tsong & Yang (1978) found that the rate of conformational changes of cytochrome P-450 increases when the lipids are in the fluid state, saturating at a lipid/enzyme ratio of 20. In addition to fluidity, reconstitution experiments have demonstrated the influence of lipid charge on protein activity, e.g. the preference of Na, K-ATPase for negatively-charged lipids (Kimelberg, 1976). However, it appears (Sandermann, 1978) that in no case has a *specific lipid* requirement been convincingly demonstrated. This suggests that the role of the surrounding lipids is limited to providing a favourable structural environment for the proteins.

The above discussion of enzyme activity naturally leads us to a consideration of lipid–protein clustering, aggregation and phase

† In such cases the distinction between integral (or intrinsic) and peripheral (or extrinsic) proteins can become ill-defined (Boggs & Moscarello, 1978*a*).

separation. We have noted how certain lipids may be bound preferentially either as a consequence of electrostatic interactions or in order to minimize the mismatch in the packing around the surfaces of proteins. The heterogeneous lipid composition of biological membranes may be necessary to satisfy these different packing requirements of proteins. This may also explain why mixed lipid phases are more efficient than a single lipid species in reconstitution studies (Sandermann, 1978). Such 'packing selectivity' should also apply to proteins. Thus the interactions between different proteins may be governed to some extent by favourable or unfavourable packing considerations, mediated by their boundary lipids (Marčelja, 1976; Israelachvili, 1977, 1978). Both attractive and repulsive forces may arise as a consequence of packing restrictions. For example, for the same reason that cholesterol is often excluded from the vicinity of proteins so may other proteins. Such effects can lead to an effective force between proteins though as yet there is no direct evidence for this. On the other hand there is now much evidence that protein aggregation is dependent on lipid phase separations. As might be expected, the phase separation of lipids into fluid and solid phases invariably leads to the segregation of proteins into the fluid domain (Höchli & Hackenbrock, 1979; Chen & Hubbell, 1973; Letellier, Moudden & Shechter, 1977). In particular, the state of aggregation of rhodopsin molecules incorporated into bilayers of various phospholipids can be altered by varying the lipid chain length and by bleaching, which has also been found to change the state of the boundary lipids (Favre *et al.* 1979). This affords an example of the direct involvement of boundary lipids in protein–protein interactions.

It is beyond our scope to review protein aggregation and cross-linking mechanisms that are induced by extra-membrane agents such as microtubules, microfilaments, ligand binding, etc.; the reader is referred to recent reviews by Berlin, Caron & Oliver (1979) and Nicolson (1976) on these topics.

Finally, we note that in all the above discussions there is an implicit assumption – the essence of the fluid mosaic model – that the structural framework of membranes is a lipid bilayer, albeit modified. This is not an essential requirement for membrane stability. There is no thermodynamic reason why proteins alone cannot self-assemble into sheets, and indeed tubulin is known to aggregate into extended sheets in certain ionic solutions (Amos, 1979).

C. *Local curvature, and interaction between membranes*

The major structural difference between natural membranes and reconstituted lipid–protein membranes such as vesicles is that the former are compositionally highly asymmetric, often possessing an actively maintained electrochemical potential gradient across the membrane. Natural membranes also have regions of different curvature, with planar and highly-folded or invaginated regions in different parts of the same membrane. Such lateral heterogeneities can lead to *lateral* electrochemical potential gradients. It is difficult to probe the importance of these structural aspects in reconstituted systems, and greater attention has to be paid to studies on intact cellular organelles.

McLaughlin & Harary (1974) have pointed out that the existence of a membrane potential will itself lead to an equilibrium distribution of charged and uncharged components that will be asymmetric; and Israelachvili (1973) has invoked similar electrostatic considerations to show that the surface charge density will be different at highly-curved regions, with a preferential migration of negatively-charged species towards the outer curved regions. In addition to these considerations the different components on each side of a real membrane must be able to pack together in accordance with the general criteria discussed previously (Carnie *et al.* 1979; Wieslander *et al.* 1980).

There are several factors that can induce membranes to curve:

(i) Many examples are known where *intra-membrane interactions* lead to local shape changes, pinocytosis, etc. Both lipid bilayers and biological membranes will in general deform when only one side is perturbed, for example, by cation binding or drug and protein insertion (Papahadjopoulos, 1977; Sheetz & Singer, 1974; Kimelberg & Papahadjopoulos, 1971). Cation binding to the inner erythrocyte membrane causes it to undergo the discocyte-to-echinocyte (spiculed) shape transformation due to the contraction of the inner half of the membrane. The preferential association of proteins and lipids in a membrane may also induce a locally-curved shape if these can pack more comfortably into a curved structure. As mentioned above cholesterol and large hydrophobic (integral) proteins may be expected to be excluded from such highly-curved regions, which would tend to accumulate more fluid-like molecules or peripheral proteins (Fig. 5.2*a*). Consistent with this is the finding (cf. Bretscher, 1976; Shukla *et al.* 1978) that microvesicles budding off from the erythrocyte plasma

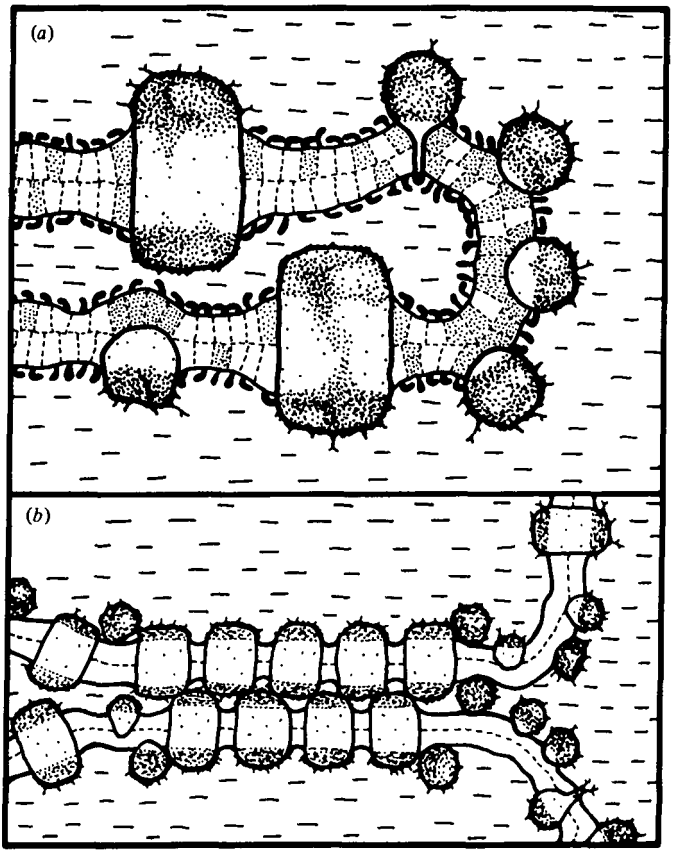

Fig. 5.2. Schematic illustration of the way lipids and proteins may be organized around (*a*) a curved membrane; and (*b*) at contacting sites.

membrane are depleted of proteins and cholesterol. The suggestion by Bretscher (1976) that a 'molecular filter' is responsible for this is unnecessary. One further interesting effect of cholesterol may also be mentioned: Nakai & Kawasaki (1959) studied the course of growing nerve fibres (filopodia), and observed that when filopodia tips reach a cholesterol droplet they quickly retract, becoming swollen and less curved. This is to be expected in view of the packing shape of cholesterol whereby any highly-curved membrane might react similarly on picking up cholesterol, especially in the outer half of the bilayer. This effect highlights the importance of the chemical environment of membranes in determining their shape.

(ii) A membrane may fold and invaginate as a consequence of such *external constraints* as a change in the osmotic balance or its confinement within a restricted space in a cell. For example, the convoluted inner mitochondrial membrane is constrained by the outer membrane. Studies on the compositional asymmetry of this membrane indicate that the tips of the cristae have a higher concentration of anionic sites (Hackenbrock & Miller, 1975). The clustering of anionic lipids and proteins at the tips of other membrane extensions and protrusions has also been reported (Grinnel, Tobleman & Hackenbrock, 1975; Anderson & Hein, 1977). These localizations of anionic sites are generally believed to be involved in cell–ligand and cell–cell interactions, Ca^{2+} binding, and transport. In addition to the affinity of anionic membrane components for the outer faces of curved membrane regions, packing considerations too lead us to conclude that only those molecules capable of packing into highly-curved regions would be found in them, whereas cholesterol and large hydrophobic proteins would be excluded.

(iii) The forces involved in *membrane–membrane interactions* that lead to such phenomena as adhesion and fusion are notable for their complexity. These can include, in order of decreasing theoretical understanding, attractive van der Waals forces, attractive or repulsive electrostatic (double-layer) forces, repulsive hydration forces, and mechanical (or polymer) bridging forces. In the only system for which detailed measurements have been reported so far – myelin membranes (Rand, Fuller & Lis, 1979) – all four forces appear to be involved.

Furthermore, as discussed in Section II the interactions *between* membranes cannot be treated in isolation from those occurring *within* membranes. Unfortunately, theoretical modelling of membrane–membrane interactions tends to ignore this interdependence which is particularly important for fluid multicomponent membranes, where each can respond to the presence of the other by a redistribution of its components to give the most favourable inter-membrane interaction (Fig. 5.2*b*). Many studies on membrane and vesicle fusion have found that proteins redistribute during the fusion process (Ahkong *et al.* 1975; Weiss, Goodenough & Goodenough, 1977; Zakai, Kulka & Loyter, 1977). Even in the absence of fusion both protein and lipid redistribution have been reported to occur at membrane–membrane contact sites. For example, the inner surface of the outer mitochondrial membrane has a non-random distribution of anionic sites, with a lower density of sites in those areas surrounding the inner membrane (Hackenbrock &

Miller, 1975). By contrast the distribution of anionic sites on the outer surface is random.

Studies on a variety of other contacting membranes also indicate a completely modified structure at membrane junctions (e.g. Hoi Sang, Saier & Ellisman, 1979) some of which contain tightly-packed crystalline arrays of particles (e.g. Peracchia, 1978; Franke *et al.* 1978). In thylakoid membranes the non-random distribution of particles between the stacked (grana) and unstacked (stroma) membrane regions is eliminated during unstacking whereupon the particles intermix randomly (Staehelin & Arntzen, 1979). These examples serve to illustrate that there exists a relation between protein aggregation within membranes and membrane coupling.

As a particular example of this it has been found (Andersson & Anderson, 1980) that in thylakoids the stacked membranes contain most of the light-harvesting photosystem II complex whereas the unstacked membranes contain the photosystem I reaction centre – a clear example of a structural determinant of membrane function.

D. *Concluding remarks*

Although membrane structure has its own intrinsic interest, it is studied primarily in order to understand its relation to biological function. Taking this relation at its most basic level, the mere *existence* of a membrane is necessary for those functions which require compartmentalization. In this review we therefore started by asking and then answering the question: when does a mixture of amphiphilic molecules form a stable, impermeable and yet flexible membrane? *Within* membranes, structural diversity is achieved by a non-random distribution of membrane components, giving rise to regions which are rigid, fluid, curved, or juxtaposed to other membranes. In addition, there are proteins playing cytoskeletal structural roles, for example, microfilaments and spectrin, which can also impart an additional degree of stability to membranes.

The proteins associated with membrane-related functions such as transport and energy transduction must necessarily be located in or near a membrane. But there is a further advantage in locating multiprotein systems in a two-dimensional membrane simply because this enhances the probability of collisions between them relative to that in solution. An example of a reaction controlled by two-dimensional diffusion is given by Liebman & Pugh (1979), who have proposed that

in the early stage of visual transduction an excited rhodopsin molecule activates many molecules of the enzyme phosphodiesterase via lateral diffusion within the rod disc membrane.

Because of the variability of membrane proteins, a different set of lipids may be required to accommodate each of them in a membrane and maintain their activity. This appears to be the role of boundary lipids, and probably explains why there is such a diversity of lipids in most biological membranes, in addition to the need for the asymmetric lipid distribution between the two sides of a membrane.

Certain protein activities, particularly those requiring collision between proteins and so limited by diffusion, can be modified by changing the protein to fluid-lipid ratio. For example, Schneider *et al.* (1980) have shown that the rate of electron transfer is limited by the rate of lateral diffusion of oxidoreduction components in the inner mitochondrial membrane, the rate decreasing proportionally to the increased lipid/protein ratio. Such modulations of the mean distance between colliding intramembrane particles can also be achieved whenever there is a lateral inhomogeneity caused, for example, by non-uniform membrane curvatures, protein interactions, and lipid phase separations. The results of Ruysschaert *et al.* (1977) indicate that lipid segregation modulates the immunological recognition in membranes, and many other examples of such structure–function relationships were described. Another possible functional advantage of the phase separation of lipids has been suggested by Shimshick & McConnell (1973): if a mixture of lipids is in the phase transition temperature range, the hydrocarbon chain region has a high lateral compressibility, and so maintains a constant lateral chain pressure when the membrane area is changed. This helps insulate the membrane against thermal or osmotic shock, and against area changes due to incorporation or removal of membrane material. Linden *et al.* (1973) have shown that sugar transport in *Escherichia coli* is enhanced in the phase separation region. Further support for the idea that it is favourable to have membrane lipids near their phase transition region comes from the observation that many organisms change their lipid composition in response to a change in ambient temperature, i.e. synthesizing more unsaturated lipids at lower temperatures, thereby stabilizing the bilayer structure.

The main criterion for membrane stability appears to be that the heterogeneous lipid mixture should be able to self-assemble into stable bilayers, even though individual species, e.g. cholesterol, may not. Thus

Cullis & Hope (1980) have suggested that elevated levels of sphingomyelin which accompany early stages of atherosclerosis may occur in response to high membrane concentrations of cholesterol. Likewise, Wieslander *et al.* (1980) have shown that the *in vivo* response of *A. laidlawii* to external stimuli, such as temperature changes and incorporation of fatty acids and cholesterol, is such that the cell regulates its membrane lipid metabolism to synthesize just those lipids that will maintain packing compatibility within the membrane.

These two examples illustrate the manner in which cells control their membrane structure. They do not do this by a crude mechanical pushing and pulling of components: lipids and proteins are not 'placed' in position, and contacting membranes are not 'pulled' apart. The cell synthesizes the right lipids and proteins, and then leaves them to do their job.

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