REVIEW ARTICLE

New biophysical techniques and their application to the study of membranes

Dennis CHAPMAN* and James A. HAYWARD*†

*Department of Biochemistry and Chemistry, Royal Free Hospital School of Medicine, London NW3 2PF, U.K., and †Department of Biochemistry, State University of New York at Stony Brook, Stony Brook, NY 11794, U.S.A.

Introduction

Our present-day views of the structure and dynamics of biological membranes have evolved through the application of biophysical methods. Recent advances in the correlation of function with biomembrane structure have followed closely upon new developments in methodology and instrumentation, particularly in the field of spectroscopy.

While much progress has been made in the study of lipid dynamics, little information is available on the structural dynamics of membrane proteins. The limits of the advances made in this field can be illustrated with the proteins involved in ion transport. The intramembraneous structure of the ionophore gramicidin A, a polypeptide only 15 amino acids in length, has been the subject of considerable debate for many years (Urry *et al.*, 1971; Veatch *et al.*, 1974; Lotz *et al.*, 1976; Nabedryk *et al.*, 1982; Wallace, 1984). Elucidation of the molecular dynamics of ion transport by gramicidin, which is perhaps the simplest model for intramembraneous proteins, is a logical first step in determining the mechanisms by which ions negotiate the dielectric barrier of biological membranes. As yet, the mechanistic proposals for gramicidin-mediated transport are largely speculative (Mackay *et al.*, 1984). The complexities exhibited by gramicidin-mediated transport are minimal, however, when contrasted with the structurally complex proteins involved in active transport of ions and in the formation of gated ion channels. To these considerations we need to add that membrane protein structures may undergo conformational changes with variations in pH, temperature, the concentration of metal ions and 'trigger' molecules and, in some cases, under the influence of light.

Recently, new techniques have been introduced into membrane biochemistry which may reveal the

influence of these environmental changes upon the dynamics of membrane proteins. Considerable advances have already accumulated from the application of these methods to investigations of the static and dynamic order of membrane lipids and their influence upon protein structure and function. The purpose of this Review is to present the insight provided by the application of new methodologies to biological and model membranes. Information on dynamic behaviour as revealed by spectroscopic studies will be considered in relation to the results obtained by static structural methods.

Generalizations on the structure of biomembranes

The fluid dynamics of membrane lipids are emphasised in the currently popular 'fluid mosaic model' of membrane structure (Singer & Nicolson, 1972). This paradigm summarizes the results of many previous investigations and envisages membrane proteins as floating in a two-dimensional lipid bilayer. The lipids and proteins are thus seen to diffuse freely within the plane of the bilayer. This emphasis on intrinsic mutability accommodates the great variety of molecular species present in biological membranes, and permits the selective modification (via protein insertion) of the bilayer permeability barrier without profound structural alterations. This model has proved useful for emphasizing the dynamic character of biological membranes, yet it is deficient in the light of many subsequent studies.

(i) Although the proteins in many biomembranes are arranged in a random fashion within the membrane plane, differentiated regions of some membranes contain specific proteins arranged in a non-random two-dimensional matrix. The spatial organisation of biomembrane components can, in fact, assume all degrees of order from an essentially complete disorder, to the quasi-crystalline order exhibited by the purple membrane (Henderson & Unwin, 1975). A recent example of the remarkably regular array which the proteins in some biomembranes can adopt is seen in the electron micro-

Abbreviations used: T_c , main gel-to-liquid crystal phase transition; T_1 , spin-lattice relaxation time; T_2 , spin-spin relaxation time; H_{11} , hexagonal phase; s.t., saturation-transfer; F.t., Fourier-transform.

graphs of whole cells and thylakoids of the phototrophic bacterium, *Rhodospseudomonas viridis*, shown in Fig. 1 (Welte *et al.*, 1981; Welte & Kreutz, 1982).

(ii) In many biomembranes the intrinsic proteins are not free to diffuse readily in the plane of the membrane, but are fixed in position as a result of either (a) high protein concentration, (b) protein aggregation (Naqvi *et al.*, 1973), (c) lipid domain formation, or (d) the interaction of the intrinsic membrane protein with underlying cytoskeleton (Nicolson, 1976).

(iii) Whilst the lipid hydrocarbon chains in biological membranes are often in a fluid, disordered state, in some biomembranes (e.g. *Acholeplasma laidlawii* and *Halobacterium halobium*) large amounts of ordered lipid are present (Steim *et al.*, 1969; Chapman & Urbina, 1971; Jackson & Sturtevant, 1978).

Despite the great variability in biomembrane functions and the inadequacies inherent in any model, some generalized features common to all biological membranes are apparent. The profiles of electron density across biomembranes exhibit a pair of high electron-density peaks which are separated by a low density trough; these characteristics are diagnostic for lipid bilayers (Blaurock & Wilkins, 1969).

Biological membranes exhibit obvious functional asymmetries in the homeostatic maintenance of their surrounded volume; the inside of any cell or organelle is different from the outside. Transport of molecules across the membrane must be directional and any given receptor, enzymically active site or immunological determinant is found on only one side of a membrane. In contrast to the absolute asymmetry of membrane proteins, membrane lipids exhibit partial asymmetry in that most lipid species are found in both halves of the bilayer, but often at different concentrations (Bretscher, 1973; Rothman & Lenard, 1977; Op den Kamp, 1979). The origins, mechanisms for maintenance, and functions of lipid asymmetry are poorly understood. Given these general comments, let us now examine some of the details of biomembrane structure and dynamics.

Lipid organization and lipid dynamics

Magnetic resonance studies

N.m.r. is an extremely powerful spectroscopic method because of its ability to focus on nuclei of a single species whilst other nuclei remain spectroscopically 'transparent'. Only those species with a net magnetic moment will interact with an imposed magnetic field; hence, ^1H , ^2H , ^{13}C , ^{14}N , ^{19}F and ^{31}P are the principal probes used in n.m.r. studies of biomembranes.

Proton resonances were employed in the early n.m.r. investigations of membranes (Chapman & Salsbury, 1966; Vekslis *et al.*, 1969). These initial studies demonstrated the increased extent of motion and rotational isomerism which occurs when phospholipid molecules are heated above the temperature of the main gel-to-liquid crystal phase transition (T_c). Selective deuteration of the fatty acid chains permitted a clear distinction of the resonances associated with the hydrocarbon and head group regions.

The principal difficulty encountered in the use of proton n.m.r., the resolution of multicomponent and overlapping spectra into assignable resonances, was eliminated by the introduction of deuterium resonances and deuterium substitution (Oldfield *et al.*, 1971). For a ^2H nucleus, there are two allowed transitions which give rise to a quadrupole splitting of the n.m.r. absorption line (Fig. 2). The separation between the two peak maxima is dependent upon the angle formed by the axis of the applied magnetic field and the principal axis of the electric field gradient tensor at the deuterium nucleus. The deuterium n.m.r. spectrum is, therefore, highly orientation-dependent; this angular dependence is conveniently expressed as the order parameter, S_{mol} . For the hydrocarbon regions of lipid bilayer systems, S_{mol} has a value of 1 for a *trans* arrangement with respect to rotation about C-C bonds, and 0 if equal proportions of *trans* and *gauche* isomers are present. Therefore, the deuterium n.m.r. resonances yield direct information about the degree of packing of the acyl chains, i.e. the static order. Such information is more difficult to obtain from n.m.r. probes which do not possess a quadrupolar moment. In addition, when contrasted with other non-n.m.r. physical probes, the deuterium label is non-perturbing. Several excellent reviews are available which provide detailed discussions of ^2H n.m.r. and its application to the study of membrane lipids and their dynamic dependence upon temperature, chain saturation, lipid-protein interactions, cholesterol and counter-ions (Seelig & Seelig, 1980; Griffin, 1981; Davis 1983; Smith & Oldfield, 1984). We provide below a brief description of several key experiments.

Although isotopic enrichment methods enable the resolution of resonances derived from different positions within a single molecular species, the interpretation of spectra arising from a distribution of inequivalent sites can be difficult. In the case of unaligned membrane samples, and for polycrystalline solids, the so-called 'powder spectrum' is obtained and consists of the superposition of the spectra contributed from molecules in every orientational plane. These spectra are inhomogeneously broadened when phospholipids are

Fig. 1. Electron micrographs of membranes of the phototrophic bacterium *Rhodospseudomonas viridis* (a) Freeze-fractured whole cells. The etched surfaces show a hexagonal lattice. The interparticle distance is 13 nm. The particles are formed by a reaction centre surrounded by several antenna-pigment complexes (from Welte *et al.*, 1981). (b) Negatively stained, isolated thylakoids. Even without image analysis, the particles are seen to consist of a central peak, surrounded by a ring. The bars represent $0.1\ \mu\text{m}$ (from Welte *et al.*, 1981).

Fig. 2. Comparison between the n.m.r. spectra obtained from a terminally deuterated phospholipid and the e.s.r. spectra obtained from a spin-labelled fatty acid (12-doxylstearate) in phosphatidylcholine

The spectra demonstrate the effects of temperature and the inclusion of a membrane protein, cytochrome oxidase, on the lipid order of the bilayer interior. The quadrupole splitting of the n.m.r. line is decreased by increased temperature or the addition of a membrane protein. In contrast, the e.s.r. spectra are similar for the liquid-crystalline lipid in the presence of the protein and for gel-state lipid. The spectra representing protein-containing samples were obtained at the same temperatures used for the liquid crystal samples (after Kang *et al.*, 1979).

perturbed by phase separations, regions of high curvature, interactions with ions, cholesterol or proteins, formation of non-bilayer structures and in mixed-membrane systems (e.g. in eukaryotic cells with multiple intracellular membranes). Most nuclei in which dipolar relaxation mechanisms dominate yield essentially featureless powder spectra (for exceptions see Sternin *et al.*, 1983), but the sharp quadrupolar splitting of the deuterium nucleus yields the characteristic powder lineshape dominated by two peaks (the 'Pake doublet'). Extraction of the oriented domains can be accomplished by a deconvolution technique, 'de-Paking', which is applicable to the membrane systems listed above (Bloom *et al.*, 1981).

Calorimetric studies demonstrated that the enthalpy changes associated with the gel-to-liquid crystal transitions of phospholipids are lower than the enthalpy changes associated with pure hydrocarbons (Phillips *et al.*, 1969), suggesting that the

hydrocarbon chains in the bilayer core are not as disordered as they are in a pure liquid hydrocarbon. Spin-label experiments (Hubbell & McConnell, 1971), which yield an order parameter directly comparable with that obtained by n.m.r., confirmed the restrained mobility of the methylene groups in the bilayer core (relative to an isotropic liquid), but indicated a gradient of flexibility that increased along the long axis of the acyl chain from the headgroup region to the hydrophobic core. Order parameters obtained with specifically deuterated phospholipids confirmed the flexibility gradient (Seelig & Seelig, 1974), although, when juxtaposed with the e.s.r. results, some detailed differences are revealed. Deuterated phospholipids yield S_{mol} values that are approximately constant until the ninth or tenth carbon atom but then drop precipitously, while the spin probes detect essentially continuous mobility and polarity gradients. The n.m.r. data also suggest an inequiv-

alence of the two acyl chains, perhaps due to a sharp bend in the first segment of the acyl chain that is esterified to the middle carbon atom of glycerol (Seelig & Seelig, 1974). This difference in alignment, which persists along the entire chain, may contribute to the increased mobility experienced by the terminal methyl group. The general profile of the flexibility gradient has been verified, by using a variety of probes and methods, in many artificial (Levine *et al.*, 1972; Stockton *et al.*, 1976; Oldfield *et al.*, 1978; Seelig & Browning, 1978; Davis *et al.*, 1979; Skarjune & Oldfield, 1979; Browning & Seelig, 1980; Seelig & Seelig, 1980) and biological (Oldfield *et al.*, 1972; Stockton *et al.*, 1977; Fretten *et al.*, 1980; Sauerheber *et al.*, 1980; Hayward *et al.*, 1982) membranes. Measurements of membrane 'microviscosity', as determined by the rate of depolarization of the fluorescence of 1,6-diphenyl-1,3,5-hexatriene, described an average hydrophobic environment (including non-membraneous environments) and are insensitive to fluidity gradients.

As opposed to static order, information about membrane dynamic order (i.e. the rate of molecular motion) is more frequently determined from n.m.r. spectra, especially for the non-deuterium probes. Measurements of relaxation times, the periods required for a system of spins to return to equilibrium after being perturbed by an applied radio frequency, yield correlation times for molecular motions. The range of spin-lattice relaxation times, T_1 , and the spin-spin relaxation times, T_2 , vary with the nuclear species; hence, the range of correlation times amenable to experimental study will also depend upon the type of n.m.r. probe employed. For deuterium probes, the window of correlation times falls conveniently within the limits of motional rates expected for membrane systems (Smith & Oldfield, 1984). Relaxation rates may be determined by analysis of ^2H spectral line widths or, more directly, by frequency-pulse methods. A powerful combination of these two approaches may be found in the quadrupolar echo Fourier transform method (see, e.g., Rice *et al.*, 1981), wherein the ^2H n.m.r. line shape is analysed as a function of the separation, in time, of two radiofrequency pulses that are out of phase with one another (Davis, 1983). Thus, ^2H n.m.r. spectra provide information about both the type of motion and the rate of motion.

T_1 relaxation measurements of ^{13}C -enriched lecithins above the T_c indicated that the lipids packed most tightly at the glycerol group (Levine *et al.*, 1972), and that motion also increased in the direction away from the glycerol moiety toward the trimethylamino group. More recently, the increased resolution afforded by high magnetic field strengths has permitted the resolution of numerous

carbon resonances using natural-abundance, proton-decoupled ^{13}C n.m.r. (Brainard & Cordes, 1981). The line widths of resonances for carbon atoms in the head group region were found to be independent of cholesterol content; this result is in strong contrast to the well-documented ordering effect of cholesterol on the acyl carbons above their transition temperature.

The individual headgroups of different phospholipid classes may be distinguished in ^2H n.m.r. spectra, and their motion appears to be relatively independent of the molecular structure in the hydrocarbon region (Smith & Oldfield, 1984). The zwitterionic dipoles of the headgroups of phosphatidylcholine and phosphatidylethanolamine lie flat within the bilayer plane (Seelig & Seelig, 1980), while the head group of galactocerebroside is essentially perpendicular to the membrane surface, projecting into the aqueous phase (Skarjune & Oldfield, 1982).

The effects of lipid unsaturation on membrane dynamics are of special interest because a large proportion of naturally occurring phospholipids are unsaturated in one (usually at the *sn*-2 position of glycerol) or both acyl chains. Measurements of the quadrupolar splittings of the variously deuterated positions of saturated and unsaturated lecithins indicate that the profile of the flexibility gradient is not appreciably affected by unsaturation (reviewed by Davis, 1983). The profiles are, however, shifted toward more disorder, consistent with the lowering of the phase transition temperature (Ladbrooke *et al.*, 1968). In contrast, the quadrupolar spin-lattice relaxation times are significantly shorter in the region of double bonds (Brown *et al.*, 1979), corresponding to increased motional rates.

Most phospholipids spontaneously form bilayers upon dispersion in aqueous media; however, a variety of non-lamellar structures may be formed with specific lipids under specific conditions. The individual structures of non-bilayer conformations may be identified unequivocally by X-ray diffraction (Luzzati & Husson, 1962). Recently, the inverted hexagonal (H_{II}) phase has been the subject of much attention because of its suggested role in secretion and membrane fusion and in transbilayer lipid transport (Cullis & de Kruijff, 1979). ^{31}P n.m.r. spectra yield an essentially diagnostic pattern for membrane systems containing hexagonal phase lipid. The added dimension of rotational motion that occurs along the cylindrical axis of the H_{II} phase gives rise to a slightly sharpened lineshape but with an axial powder pattern that is in the sense opposite to that of the lamellar powder pattern (Cullis & de Kruijff, 1978). In the presence of biphasic (hexagonal-lamellar) systems, it is difficult to separate quanti-

tatively the components of the ^{31}P spectra. 'De-Packing' of these spectra will permit resolution of the spectral properties of each phase, and the integrated intensities would provide a measure of their relative amounts (Sternin *et al.*, 1983). This type of quantification is a necessary prerequisite to the unequivocal assignment of biological relevance for hexagonal phase lipids.

Perhaps the best studied component of mammalian membranes, cholesterol is the most abundant sterol in animal tissues and an important determinant of their fluid properties and permeability. Its amphiphilic structure consists of a hydroxyl group oriented at the aqueous interface and capable of hydrogen bonding to the glycerol ester oxygen of phospholipids, a rigid planar ring that renders a monomolecular film virtually incompressible (Chapman *et al.*, 1969), and a short alkyl side chain that may contribute to the increased fluidity of the membrane interior (Taylor & Smith, 1980). Early ^{13}C n.m.r. studies of model membranes performed by Opella *et al.* (1976) indicated that the isopropyl section of the side chain had considerable rotational freedom, while the sterol ring itself underwent highly anisotropic motion. Results obtained by deuterium n.m.r. (Oldfield *et al.*, 1978) indicate that the sterol molecule undergoes rapid axial rotation (greater than 10^7 s^{-1}), together with a slight off-axis 'wobble', which vary as a function of temperature and lipid bilayer composition. Many studies have been made of the modulation of lipid chain ordering by the presence of cholesterol in lipid bilayers. The initial proton n.m.r. studies (Chapman & Penket, 1966) demonstrated that, above the T_c , addition of cholesterol inhibits the motion of the hydrocarbon chains of fluid lipid bilayers. In the ^2H n.m.r. experiment, this results in a 2-fold increase in the quadrupolar splitting at equimolar ratios of cholesterol to phosphatidylcholine (Oldfield & Chapman, 1972; Rice *et al.*, 1979). Below the T_c , however, cholesterol prevents lipid hydrocarbon from crystallizing into the more ordered crystalline or gel phases. The latter effect is seen as a maintenance of the 50 kHz quadrupolar splitting in the presence of cholesterol upon cooling below the T_c as compared with the broad, featureless spectrum that is obtained for gel-state phospholipids in the absence of cholesterol. Below -60°C , the spectra both with and without cholesterol appear rather similar; the breadth and shape of both spectra suggest that the only motion now occurring is slow axial diffusion.

The advantages of spin label techniques and e.s.r. spectroscopy are a high sensitivity and a capacity to observe motion occurring over a wide biologically relevant time scale [10^{-11} – 10^{-7} s for conventional e.s.r. and 10^{-7} – 10^{-3} s for saturation-transfer (s.t.) methods]. The spin label approach

requires the addition of an exogenous spin label, and therefore risks perturbation of membrane structures by the probe. Nitroxide spin probes are most frequently employed in membrane studies; the small size of the functional group may limit its perturbation of membrane structure. The method is in its third decade of application to the study of membrane dynamics, and many detailed reviews are available (e.g. Gaffney, 1974; Berliner, 1976, 1979; Marsh, 1981; Marsh & Watts, 1982). A formal description of e.s.r. lineshapes is beyond the scope of this Review. It is worth noting, however, that the appearance of the nitroxide spectrum (Fig. 2) is determined, in part, by: (a) the polarity of the environment surrounding the probe, (b) the type of motion exhibited by the probe, (c) the rate of motion, (d) the orientation of the probe (actually the π orbital in which the unpaired electron resides) with respect to the magnetic field, and (e) the extent of paramagnetic interactions.

The polarity-dependence of the e.s.r. spectra of nitroxide-labelled fatty acids and phospholipids, reflected in the coupling constant which is derived from the distance (in G) separating the hyperfine peaks, has been utilized to examine the membrane polarity profile in chromaffin granules (Fretten *et al.*, 1980) and platelets (Hayward *et al.*, 1982). In contrast with the flexibility gradient reported by spin probes, membrane polarity reaches a minimum value at approximately the twelfth carbon atom, and is essentially constant to the end of the acyl chain.

Barring their dependence upon the frequency of motion, nitroxide linewidths have an especial utility in estimating the degree of paramagnetic interactions. Such interactions occur when spin labels are homogeneously distributed at high concentrations (usually taken for membrane probes as greater than 1 molecule of spin label/100 molecules of lipid), but may occur at lower probe concentrations if the distribution of the probe is not homogeneous. This capacity to detect inhomogeneous distributions of the probe has been exploited in the study of phase separations in model and biological membranes, and was the basis for the study of lipid diffusion (e.g. Scandella *et al.*, 1972) in biological membranes.

Gains in spectral resolution and detectability may also be achieved by isotopic substitution of e.s.r. probes. Perdeuterated nitroxides yield signal-intensity enhancements of up to 5-fold; a further 5-fold enhancement results from ^{15}N substitution of the deuterated analogue (Beth *et al.*, 1981). These probes should have significant advantages in membrane studies wherein lineshapes may be distorted by sequestration of the probe. Under these circumstances, the utility of lower probe concentrations rests in the limitation of paramag-

netic interactions. Recently, it has become possible to analyse the intramembraneous collision frequencies between unlike molecules by the use of ^{14}N and ^{15}N spin probes (Davoust *et al.*, 1983a,b). Thus, it has been demonstrated that, although a probe attached near the methyl terminus of a fatty acid explores a large volume, it collides with very low probability with a probe attached near the head-group region of a phospholipid. In contrast, the similarly labelled methyl ester does collide frequently with the polar head groups, indicating that the methyl ester is not as stringently positioned within the membrane. Hence, spin-labelled fatty acids, but not the corresponding esters, may be employed to investigate the effects of perturbants on membrane structure. Whenever possible, the phospholipid spin analogues should be used in order to obtain the best representation of the behaviour of membrane phospholipids.

Lipid motions in membranes are, in general, sufficiently rapid to enable their analysis by conventional e.s.r. methods. However, the development of saturation-transfer methods (Thomas *et al.*, 1976) has permitted detailed analyses of slower motional regimes, and has particular application to the study of membrane proteins and motionally restricted membrane lipids. The method enables characterization of different types of motion; rotational diffusion along the different nitroxide axes may be independently assessed. Quantitative analysis of anisotropic motion, however, is limited due to the deficiency of theoretical simulations of the effects of anisotropic motion on s.t.-e.s.r. spectra, although these studies are gradually progressing (Robinson & Dalton, 1980; Johnson *et al.*, 1982). Notwithstanding these limitations, s.t.-e.s.r. may be quite effectively utilized as a sensitive probe for changes in molecular dynamics, particularly for lipids in the gel phase. For a variety of phospholipids in their gel phase, the effective correlation times determined by s.t.-e.s.r. for motion of the long molecular axis were approx. 10^{-4} s (Marsh & Watts, 1980). The co-operative onset of a rapid, anisotropic long-axis rotation of phospholipids was demonstrated to coincide with the pretransitions of phosphatidylcholine- and phosphatidylglycerol (Watts & Marsh, 1981). Those phospholipids which exhibit a pretransition are characterised by an increase in rotational correlation times of roughly two orders of magnitude when heated through the pretransition.

Other spectroscopic methods

Formerly the domain of chemical spectroscopy, i.r. spectroscopy has found increasing application to biological systems because computer methods permit the subtraction of the overlapping water absorbance in aqueous samples. Dispersive and

Fourier-transform (F.t.) i.r. spectroscopy provide non-perturbing measurements that are sensitive to motion on time scales more rapid than n.m.r. or e.s.r. (reviewed by Fringeli & Günthard, 1981; Amey & Chapman, 1983). The impact of F.t. methods on i.r. spectroscopy is essentially similar to that observed for n.m.r. in the 1970's. Spectra are signal-averaged in order to increase the signal-to-noise ratio. Presently, complete spectra may be obtained at the rate of approximately 1/s, but it is likely that developments in instrumentation will permit spectra to be recorded at an even faster rate.

The more commonly used i.r. spectra parameters are the frequency maxima and the half-band widths of the individual vibrational modes. Frequency maxima are determined by the nature of the vibrational mode and the vibrating group. I.r. analyses of phospholipid/water systems have shown that the gel-to-liquid crystal transition is accompanied by an abrupt change in the methylene band parameters (Asher & Levin, 1977; Cameron *et al.*, 1980; Cortijo & Chapman, 1981). The C-H and C- ^2H stretching frequencies provide information on the proportions of *trans* and *gauche* rotamers and have been used to monitor changes in lipid conformation. The temperature profiles of the band-maximum frequencies for the methylene stretching vibrations of dimyristoyl- and dipalmitoyl-phosphatidylcholine are shown in Fig. 3. This method detects co-operative transitions at 23°C and 41°C, respectively, in agreement with the results obtained by a variety of other physical methods.

The widths of the individual i.r. contours result from rotational, translational and/or collisional effects. Thus, the half-band widths monitor the freedom of motion within the immediate environment of the absorbing group. The half-band widths are sensitive indicators of the changes that accompany lipid phase transitions, but they are also sensitive to other changes that do not introduce *gauche* conformers, such as a decreased freedom of librational or torsional motion (Casal *et al.*, 1980). I.r. spectra sense the lamellar-hexagonal transition of phosphatidylethanolamine (Mantsch *et al.*, 1981), and show evidence of increased conformational and motional disorder in the acyl chains when present in the hexagonal phase. Perturbations by cholesterol may also be observed by the i.r. method. Elevation of the cholesterol content increases the number of *gauche* conformers (i.e. decreases the order) below T_c , and decreases the number of *gauche* conformers (i.e. increases the order) above the T_c . The magnitude of these effects increases with cholesterol concentration. At very high cholesterol concentrations, there is almost no temperature-dependent change in the relative populations of the two conformers.

Fig. 3. Temperature-dependence of the maximum wave number of the CH_2 asymmetric stretching vibrations in (a) dipalmitoylphosphatidylcholine/cholesterol (DPPC) and (b) dimyristoylphosphatidylcholine/cholesterol (DMPC) at the molar ratios indicated

The temperature-dependence for the pure lipids is also given (from Cortijo *et al.*, 1982).

Cameron *et al.* (1980) have used F.t.-i.r. spectroscopy to study the pretransition observed in calorimetric studies of saturated lecithins. The packing of the acyl chains was monitored by the spectral parameters for the methylene scissoring, rocking and wagging modes. When heated through the pretransition, there was a progressive increase in the rates of axial motions. Most interesting, however, were the spectral changes consistent with a crystal-lattice phase change from an orthorhombic subcell (below the pretransition) to a hexagonal subcell (above the pretransition). More recently, these investigators (Cameron *et al.*, 1983) have examined the effects of membrane isolation on the thermal behaviour of phospholipids from *Acholeplasma laidlawii*. Growth of this bacterium in a medium supplemented with perdeuterated myristic acid and an inhibitor of endogenous fatty acid synthesis permits the enrichment (up to 95%) of the phospholipid fraction. These deuterium-labelled biomembranes provide a means of resolving the $\text{C}-^2\text{H}$ stretching absorption band from the $\text{C}-\text{H}$ bands arising from endogenous proteins (cf. Lee *et al.*, 1984). Analysis of the temperature dependence of the $\text{C}-^2\text{H}$ stretching bands of *A. laidlawii* confirmed the previous observation (Chapman & Urbina, 1971) that the membranes of these cells are highly ordered at the growth temperature, and

that only a small proportion of the lipid is in the liquid-crystalline state. However, in the isolated membranes a much higher proportion of the lipids were in the fluid state at the growth temperature, indicating that the conformational order of membrane lipids may be altered by the isolation process.

Additional i.r. bands may be used for probing the polar region of the membrane; the bulk of the acyl groups are electronically decoupled from the polar head groups so that perturbations in the two membrane regions may be well resolved. The carbonyl stretching bands of phospholipids are composed of two vibrational components which may be unambiguously assigned to either the *sn*-1 or *sn*-2 position (Levin *et al.*, 1982). These assignments permit investigations of conformational rearrangements within the interfacial region of phospholipid bilayers. Addition of cholesterol to pure phosphatidylcholine systems appear to reduce the conformational inequivalence between the two carbonyl groups by specifically perturbing the *sn*-2 chain (Bush *et al.*, 1980). Other vibrational modes associated with the polar head group include C-N and phosphate stretching. Fringeli & Günthard (1981) have used polarization i.r. and orientated phospholipid multilayers to examine the crystallinity in the polar region of a homologous series of phosphatidylethanolamine through phosphatidylcholine. Crystalline substances yield sharp absorption bands with strong polarization effects. Increasing the number of *N*-methyl groups decreased the crystallinity of the polar portion of the molecule, consistent with the lower T_c values observed for phosphatidylcholines.

Membrane protein structures and dynamics

Static studies of protein structures

Crystallographic techniques, which reveal the greatest structural detail for soluble proteins, are now being applied to the problem of membrane protein structure. A major problem in the application of these techniques to membranes is that integral proteins are in contact with both polar and non-polar environments. This bi-polar environment must be reproduced in the crystal in order to maintain conformation and thereby complicates crystallization (see Michel, 1983, for a discussion of crystallization of membrane proteins). As yet, only matrix porin (Garavito & Rosenbusch, 1980) and photosynthetic reaction centres from a purple bacterium (Michel, 1982) have been crystallized in forms suitable for high resolution analyses.

Matrix porin, a major component of the outer membrane of *Escherichia coli* (Di Rienzo *et al.*, 1978) in which it forms ordered, two-dimensional arrays, was crystallized from detergent solutions

(Garavito *et al.*, 1983). Large quantities of detergent remained associated with the crystalline protein; however, the structural resolution was to within 0.29 nm and reproduced the hexagonal arrangement found for the protein in phospholipid bilayers (Dorset *et al.*, 1983). Much more complex crystals were obtained for the reaction centres of *Rhodopseudomonas viridis* (Michel, 1982). The isolated and crystallized complexes contain four different protein subunits. The complexity of this reaction centre, two copies of which are present in each asymmetric unit of the tetragonal unit cell, poses a formidable crystallographic challenge. Three-dimensional crystals of bacteriorhodopsin have also been obtained (Henderson & Shotton, 1980; Michel & Oesterhelt, 1980), although their small size and the presence of structural defects preclude high-resolution X-ray crystallographic studies. The results of crystallographic attempts thus far suggest that only those proteins known to form two-dimensional crystal-like arrays within membranes have a sufficient propensity towards self-ordering to form three-dimensional crystals.

For those proteins that do not form ordered arrays, neither in three dimensions nor within the membrane plane, X-ray diffraction can be used only to obtain a profile of electron density in the direction perpendicular to the membrane plane. Such profiles are generally prepared from wet pellets. A large number of membranes, especially those specialized membrane systems in which a single protein predominates (such as myelin, purple membranes of halobacteria, vesicles of sarcoplasmic reticulum, rod outer segments and clathrin-coated vesicles), have been characterized by these methods (reviewed by Makowski & Li, 1983). The results are generally interpreted in terms of protein disposition relative to the lipid bilayer. They have also been used to determine the dimensions of protein subunits that project beyond the membrane surface [e.g. the calcium ATPase of sarcoplasmic reticulum (Brady *et al.*, 1982)].

In the absence of three-dimensional crystals, a great deal of effort has been focused on the study of preformed two-dimensional arrays of membrane proteins. Some of these arrays appear to occur naturally in differentiated membrane regions (e.g. bacteriorhodopsin in purple membrane) while others have been induced in model systems [e.g. vesicles isolated from sarcoplasmic reticulum (Dux & Martonosi, 1983a,b) and acetylcholine receptor (Klymkowsky & Stroud, 1979)]. The diffraction of X-rays, which are scattered by electrons, is not possible with such a thin crystal. Under these circumstances, diffraction patterns are best obtained from neutron and electrons, for which the scattering centres are the atomic nuclei. The three-dimensional image reconstruction of the purple

membrane, obtained by Fourier analysis of electron micrographs prepared from unstained samples, has contributed more to our knowledge of the static structure of a membrane protein than any other single method (Henderson & Unwin, 1975; Unwin & Henderson, 1975).

The amount of structural detail revealed by this method is dependent on structural preservation of the sample while in the electron beam. The use of electron-opaque stains must be avoided in order to access the non-surface structure of the protein. Extremely low electron doses limit the damage induced by the electron beam. The damage associated with dehydration of membranes *in vacuo* is prevented by immersion of the sample in a glucose solution before dehydration. This is essential, as X-ray diffraction patterns showed that drying causes shrinkage as well as disordering (Blaurock, 1975) of the purple membrane lattice. The reconstructed images are extracted from a combination of micrographs and electron diffraction patterns. Fourier analyses of the electron micrographs provide the phases of the Fourier components of the structures while the corresponding amplitudes are derived from the electron diffraction patterns. Contour maps show the atomic densities of the membrane protein in projection; a three-dimensional map is derived from serial contours obtained by tilting the specimen in the electron beam.

The combined map for bacteriorhodopsin contained seven rods of density, 3.5–4.0 nm long (i.e. sufficient to extend completely through the membrane), which may be recognized as the α -helical segments identified by X-ray diffraction (Henderson, 1975). Three of the rods are perpendicular to the plane of the lipid bilayer, while the other four are tilted slightly. Their relative tilts are consistent with a structural stabilization by helix-helix interactions; this notion finds support in the capacity to regenerate the native structure from proteolytic fragments of bacteriorhodopsin (Liao *et al.*, 1983). Attempts have been made to fit the amino acid sequence (Ovchinnikov *et al.*, 1979; Khorana *et al.*, 1979) to the three-dimensional structure (Engelman *et al.*, 1980; Jap *et al.*, 1983) and a better resolved projection map has been obtained (Hayward & Stroud, 1981). Despite these advances, the available data do not permit an unequivocal description of the structure of this protein nor its mechanism of proton translocation.

The necessity of a well-ordered, two-dimensional lattice has limited the number of structures studied by these techniques. Three-dimensional reconstructions have also been prepared for negatively stained gap junctions (Unwin & Zampighi, 1982), crystalline lattices of cytochrome oxidase (Deatherage *et al.*, 1982a,b), the acetylcholine

receptor (Ross *et al.*, 1977; Kistler & Stroud, 1981; Kistler *et al.*, 1982), and reconstituted vesicles of matrix porin (Dorset *et al.*, 1984). Micrographs of negatively-stained specimens yield information about the stain-excluding portions of the structures. Crystallographic analyses of protein structures reflect static structures; positional assignments of individual amino-acid sidechains must await improvements in the isolation of three-dimensional crystals. When interpreted in the light of crystallographic results, dynamic spectroscopic studies may soon provide detailed views of the structural fluctuations which are associated with the function of membrane proteins.

Early i.r. studies (Susi *et al.*, 1967; Timasheff *et al.*, 1967) of the amide I bands demonstrated the sensitivity of the band maximum frequency to the secondary conformation of proteins. Measurements performed in H_2O and $^2\text{H}_2\text{O}$ discriminate among α -helical, parallel and anti-parallel β -sheet and random coil conformations. Investigations of the purple membrane using conventional and polarized i.r. spectroscopy have focused on the helical nature of the protein and its orientation relative to the membrane plane (Rothschild & Clark, 1979; Cortijo *et al.*, 1982; Krimm & Dwivedi, 1982). The unusually high frequency of the amide I band suggests some distortion of the transmembrane helices (Rothschild & Clark, 1979; Cortijo *et al.*, 1982), and has been interpreted to represent the tilting of the peptide groups away from the helical axis (Krimm & Dwivedi, 1982). The presence of appreciable β -sheet conformation is indicated by the amide I shoulder at 1639cm^{-1} (Cortijo *et al.*, 1982; Jap *et al.*, 1983), and is supported by measurements of u.v. circular dichroism (Jap *et al.*, 1983). Our second-derivative i.r. analysis (Lee *et al.*, 1985) reveals a contribution at 1635cm^{-1} that may be assigned to β -sheet structure (Fig. 4). Jap *et al.* (1983) have proposed that the accumulated spectroscopic evidence is more supportive of a structural model consisting of five helices and four strands of β -sheet.

Glycophorin (MN glycoprotein) is the major glycoprotein of the human erythrocyte membrane and is composed of 131 amino acid residues with 16 attached oligosaccharide chains (Segrest *et al.*, 1973; Tomita *et al.*, 1978). The *N*-terminus has been shown to contain a stretch of about 30 residues with a high proportion of hydrophobic amino acids and not a single charged residue (Segrest *et al.*, 1973). A recent F.t.-i.r. spectroscopic investigation (Mendelsohn *et al.*, 1984) of glycophorin reconstituted in phosphatidylserine has revealed the presence of significant β -sheet conformation (10% of total). The major vibration of the amide I band occurred at 1653cm^{-1} , indicating a primarily α -helical and random coil

Fig. 4. (a) Difference i.r. spectrum of purple membrane in $^2\text{H}_2\text{O}$ buffer after subtraction of $^2\text{H}_2\text{O}$ buffer background absorption, showing the amide I band of bacteriorhodopsin, and (b) second-derivative spectrum of purple membrane from (a).

(b) reveals the band at 1635cm^{-1} which is attributed to β -structure. (D. C. Lee, J. A. Hayward, C. J. Restall & D. Chapman, unpublished work.)

backbone. This study supports an earlier suggestion (Schulte & Marchesi, 1979), based upon c.d., that the protein contains 27% α -helix, 10% β -sheet and 63% random coil conformations.

Dynamic studies of protein structures

Apart from those methodologies which reflect the rates of protein diffusion, there are few techniques available that are sensitive to the alterations in protein structure that accompany the processes mediated by membrane proteins. In order to elucidate the mechanisms by which membrane proteins function, molecular rearrangements within the structure of the protein must be investigated. Bacteriorhodopsin offers a unique system for kinetic analysis because (a) its amino acid sequence is known and its static structure is perhaps the best-characterized of all membrane proteins, (b) its activity is light-driven, eliminating the need to correct for the presence of activating molecules or triggers, (c) the chromophore (retinal) provides an endogenous indicator of the distinct stages in proton transport, and (d) the decay of the reaction intermediates is inhibited by low temperature.

Conformational changes, in both the retinal and the protein, have been detected during the photocycle of bacteriorhodopsin by F.t.-i.r. (Rothschild & Marrero, 1982). The assignment of spectral peaks to specific protein and chromophore groups was

made possible by comparison with resonance Raman spectra which reflect only chromophore vibrations (Rothschild & Marrero, 1982). Identifications of the chromophore vibrations in the F.t.-i.r. spectra were confirmed by the similarity in the peak frequencies in the Raman spectra and the changes in both types of spectra that accompanied deuteration of the purple membrane. Comparisons between F.t.-i.r. and Raman spectra for retinylidene model compounds (Siebert & Mäntele, 1980) permitted identification of the C=N stretch mode (approx. 1650 cm^{-1}) for the Schiff base formed by the linkage of retinal to a lysine residue. Cyclical shifts in the frequency of the C=N vibration, which correlated with the specific stage of the various frozen reaction intermediates, were due to alterations in the protonation state of the Schiff base (Rothschild & Marrero, 1982). Furthermore, the first intermediate (K610, where the number refers to the absorption maximum) displayed a shift of the protonated C=N vibration to lower frequency, which was interpreted as due to an increase in the distance between the Schiff base proton and its counterion. A simple model of proton translocation was proposed in which the first step of the photocycle involved a movement of the Schiff base proton away from a counterion. The appearance of a band at 1760 cm^{-1} upon formation of the last (M412) intermediate in the photocycle provided evidence for protonation of a protein carboxylate residue. At this stage of the photocycle, the Schiff base is unprotonated (Bagley *et al.*, 1982), suggesting a direct kinetic link for proton movement between the chromophore and the protein in the last stage of the photocycle.

Kinetic i.r. spectroscopy has been applied to bacteriorhodopsin and the results discussed in terms of a possible involvement of carboxylate groups in the proton-pumping mechanism (Siebert *et al.*, 1982). The sample is excited by a xenon flash and absorption changes measured by an i.r. beam. The resulting i.r. difference spectra reveal conformational changes of the i.r. active groups. Isotopic (^2H) labelling of retinal was used to identify chromophore bands and to distinguish them from protein bands. Protonation of two carboxylate residues was monitored by a change in the kinetic signal over the range $1735\text{--}1775\text{ cm}^{-1}$. Using this method, the kinetics for the rise and decay of one of the protonated states could be correlated with the rise and decay of the M412 intermediate, and was assumed to represent the counterion for the Schiff base discussed above.

The dynamic structure of bacteriorhodopsin has also been studied by ^2H n.m.r. following the biosynthetic incorporation of ^2H -labelled amino acids (Keniry *et al.*, 1984). The spectra consistently exhibited two components, a sharp central compo-

nent and a broad powder pattern, although the relative intensity of the two components varied widely. The authors ascribed 90–95% of the intensity of the central component to surface residues undergoing fast isotropic motion at the membrane surface. Essentially crystalline amino acids were made manifest by the spectral components with large quadrupolar splittings due to motion constrained by the bilayer and/or the interaction between helices or between protein molecules. The experimentally obtained percentage of residues located on the surface was correlated with the value predicted by various theoretical models proposed by other authors. All of the models yielded significant correlations with the experimental data, but specific models gave better fits than others. Aggregated sheets of purple membrane did not exhibit a sharp component, indicating that the surface residues had become immobilized. In sum, these experiments demonstrate that the dynamic structure of membrane proteins is sensitive to its environment and, by extension, to physiological changes in state.

Protein rotational and lateral diffusion

The concept of rapid diffusion of membrane proteins, which was emphasized in the early models of membrane structure, was supported by a study of the visual pigment of retinal disc membranes (Cone, 1972). Rhodopsin contains the chromophore, 11-*cis*-retinal, shared by bacteriorhodopsin. The presence of this chromophore and its arrangement within the membrane plane permits study of its rotational diffusion by the rate of decay of dichroism induced by a flash of polarized light. Cone (1972) showed that the correlation time for rotational diffusion of rhodopsin was about $20\mu\text{s}$, indicating that the protein was highly mobile. Within a year, however, similar studies with bacteriorhodopsin (Naqvi *et al.*, 1973) showed that in the purple membrane this protein yielded correlation times about 1000 times longer, i.e. it was active yet immobile. This result clearly indicated that protein mobility is not essential for function.

Naqvi *et al.* (1973) also pointed to a new technique that permits the analysis of rotational diffusion for proteins that do not possess an intrinsic chromophore. This involved the attachment of an artificial probe, such as eosin, to the protein of interest. Today, most procedures for measuring rotational diffusion utilize triplet probes, the most useful ones being the halogenated derivatives of fluorescein, tetrabromofluorescein and tetraiodofluorescein. These probes resist photodecomposition and have a high quantum yield for formation of the triplet state. For measurements of lateral diffusion, probe molecules

are used which decompose irreversibly after a bleaching pulse of high-intensity light. This feature is best exemplified by fluorescein and rhodamine. Time-resolved laser-flash (Cherry *et al.*, 1976; Hoffman *et al.*, 1979) and time-averaged (Murray *et al.*, 1983) methods have been employed to study the diffusion of a variety of membrane proteins (reviewed by Hoffman & Restall, 1983).

A detailed examination of rotational diffusion has recently been made for the $\text{Ca}^{2+} + \text{Mg}^{2+}$ -ATPase from rabbit sarcoplasmic reticulum (Restall *et al.*, 1985a). Curve-fitting analyses of both the total emission and anisotropy decay revealed complex kinetics inconsistent with a simple model for protein movement. The simplest model consistent with the experimental data requires four exponential components to define the decay of the total emission. The anisotropy decay can be interpreted by a model in which the phosphorescent probe, or a small protein segment on which the probe is located, reorients on a sub- μs time-scale about an axis attached to a larger segment. The larger segment reorients on a time-scale of a few μs about an axis fixed within the frame of the ATPase. A fraction of the protein molecules are rotationally immobile on a sub-ms time-scale, while the rest rotate on a time scale of 100–200 μs about the normal to the lipid bilayer. Addition of an excess of calcium ions has been found to cause a 20% decrease in the time-averaged phosphorescence emission anisotropy over a range of temperatures (Fig. 5; Restall *et al.*, 1985b). Anisotropy decreases with increasing temperature

because of the enhanced fluidity of the bilayer. Under these conditions, no effect of calcium on the lipid fluidity was detected by measurements of the fluorescence depolarization of the lipid probe, diphenylhexatriene. The results suggest calcium-mediated change in the conformation of the calcium pump.

The mobility of this calcium pump has also been studied by s.t.-e.s.r. (Hidalgo *et al.*, 1978) using vesicles of sarcoplasmic reticulum, and yielded correlation times of 60 μs at 4°C. Mobility of the protein was eliminated by cross-linking with glutaraldehyde. A similar result was obtained for the reconstituted protein in a pure phospholipid below its T_c , conditions under which enzyme activity is strongly inhibited.

Measurements of the lateral and rotational diffusion of proteins in biomembranes provide good evidence that, from a dynamic point of view, biological membranes exist in different states ranging from 'rigid', through 'intermediate' to 'free'. Rigid systems, such as bacteriorhodopsin in the purple membrane, are typically constrained by the formation of well-ordered protein arrays or by the viscosity of the surrounding lipid. Intermediate systems may be characterized by peripheral molecular assemblies, adjacent to the plasma membrane, which anchor the intramembraneous protein and thereby inhibit its motion. When reconstituted in defined lipid systems (above their T_c) in which peripheral membrane assemblies are absent, an increase in their rotational mobility is expected. Free systems may be characterized by the lack of additional membrane structures and a fluid lipid phase.

Fig. 5. Temperature-dependence of the phosphorescence anisotropy of erythrosin-labelled $\text{Ca}^{2+} + \text{Mg}^{2+}$ -ATPase as measured by a time-averaged technique (Murray *et al.*, 1983)

Addition (lower curve) of a 0.05 mM excess of Ca^{2+} to a sample of labelled sarcoplasmic reticulum containing EGTA (upper curve) decreases the anisotropy reported at all of the temperatures investigated (Restall *et al.*, 1985b).

Protein-lipid dynamics

The potential interactions between membrane lipids and integral proteins and the degree to which integral proteins can perturb a lipid bilayer structure have been the subject of much discussion (Gennis & Jonas, 1977; Chapman *et al.*, 1979). Early e.s.r. experiments (Jost *et al.*, 1973) lead to a model in which a single layer of lipid molecules was motionally restricted by interfacial interactions with membrane proteins whilst results obtained by i.r. (Cortijo *et al.*, 1982; Dluhy *et al.*, 1983; Lee *et al.*, 1984) demonstrated that there is no increase in chain order (i.e. no decrease in the C–H or C– ^2H stretching frequency) induced by the presence of integral proteins in lipid dispersions above their T_c . Several ^2H -n.m.r. studies of model systems (Oldfield *et al.*, 1978; Rice *et al.*, 1979; Paddy *et al.*, 1981) have indicated that a variety of proteins do not order the lipid. The observed ^2H -n.m.r. spectra of protein-lipid recombinants are virtually identi-

cal with those obtained for pure lipid bilayers or for intact cell membranes (Kang *et al.*, 1979; Gally *et al.*, 1980). Moreover, the ^2H -n.m.r. results and recent e.s.r. results (Paddy & Dahlquist, 1982) suggest that in many systems there is rapid exchange 10^6 – 10^7 s^{-1} or greater) between the bulk bilayer lipid and the lipid adjacent to the protein surface. This rate of exchange is comparable with the rate for lateral diffusion in fluid bilayers (Scandella *et al.*, 1972).

The discrepancy between the e.s.r. and n.m.r. results may be ascribed to differences in the time-scale limits of the two methods. Motion fast on the n.m.r. time-scale may be slow on the e.s.r. time-scale. Detailed theoretical analyses (Hoffman *et al.*, 1981; Pink *et al.*, 1984) have reconsidered the results obtained by probe (e.s.r. and fluorescence) methods for delipidated sarcoplasmic reticulum and recombinant systems containing the calcium pump. These studies conclude that (i) the ATPase oligomers are randomly distributed in the fluid phase, (ii) oligomers may contact each other at all concentrations, implying that there is no permanent annulus of lipid surrounding the protein, and (iii) the motion of probe molecules may be restricted by proximity to protein, but the motion of unlabelled hydrocarbon chains is not affected. The picture that emerges is one in which there are no long-lived lipid molecules surrounding membrane proteins above the T_c . Lipid molecules are seen to exist in a highly dynamic state, and collide with the irregular surfaces of proteins at rapid (10^6 – 10^7 s^{-1}) rates. An alternative interpretation has been proposed by Marsh (1983).

Conclusions

The bilayer arrangement of lipids is perhaps the only organizational feature that is common to all biological membranes. Despite the regularity of its structure, it appears remarkably mutable and may be described by a broad range of dynamic states. For technological reasons, lipid structure and dynamics have been the focus of most biophysical studies over the last two decades. However, new developments, particularly in the fields of spectroscopy, permit examination of the more biochemically relevant structures, membrane proteins. An appreciation of mechanistic proposals for the activity of membrane proteins is greatly enhanced by the determination of its molecular structure at high resolution. Combined studies, utilizing some of the methods we have outlined for determining the static and dynamic structure of membrane proteins, hold great promise for the delineation of enzyme and transport mechanisms and their relationship with membrane lipid composition.

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