REVIEW ARTICLE
Magnetic resonance of membranes

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INTRODUCTION

Membranes occupy a key position in cell biology, and understanding the molecular basis of membrane function presents a major challenge. Biochemical studies have revealed that the composition of biological membranes is complex and characteristic of a particular cell or organelle. The basic structural unit is a bilayer lamellar sheet, approx. 4–5 nm in thickness, comprising polar lipids together with neutral lipids such as cholesterol. Proteins are both embedded in the bilayer (integral) and bound at the surface (peripheral); membrane proteins, sometimes with attached carbohydrate chains, have specific functions in substrate transport, signal reception, catalysis and energy conservation. The polar lipids in biological membranes differ widely in their headgroup, backbone and acyl chain composition and there is asymmetry in the distribution of both the lipids and the proteins in the membrane. Broadly speaking, the lipid bilayer provides a vectorial matrix with specific physicochemical and reactivity characteristics which are matched to the diverse functions of biological membranes.

The above picture of biological membranes is a static one. It is well known that the functions of most membranes require mobility in the protein and lipid components as described by the 'fluid mosaic' model (Singer & Nicholson, 1972). The different types of molecular motion present in biological membranes are indicated in Fig. 1. The lipid molecules can rotate about their long axes, move laterally within the bilayer sheet, and some can undergo transverse motion ('flip-flop') across the bilayer; the lipid polar headgroups and acyl chains are conformationally flexible; the proteins rotate axially and undergo lateral motion in the bilayer plane. Much of this information about membrane dynamics has been accumulated over the past 25 years using spectroscopic methods, particularly magnetic resonance spectroscopy.

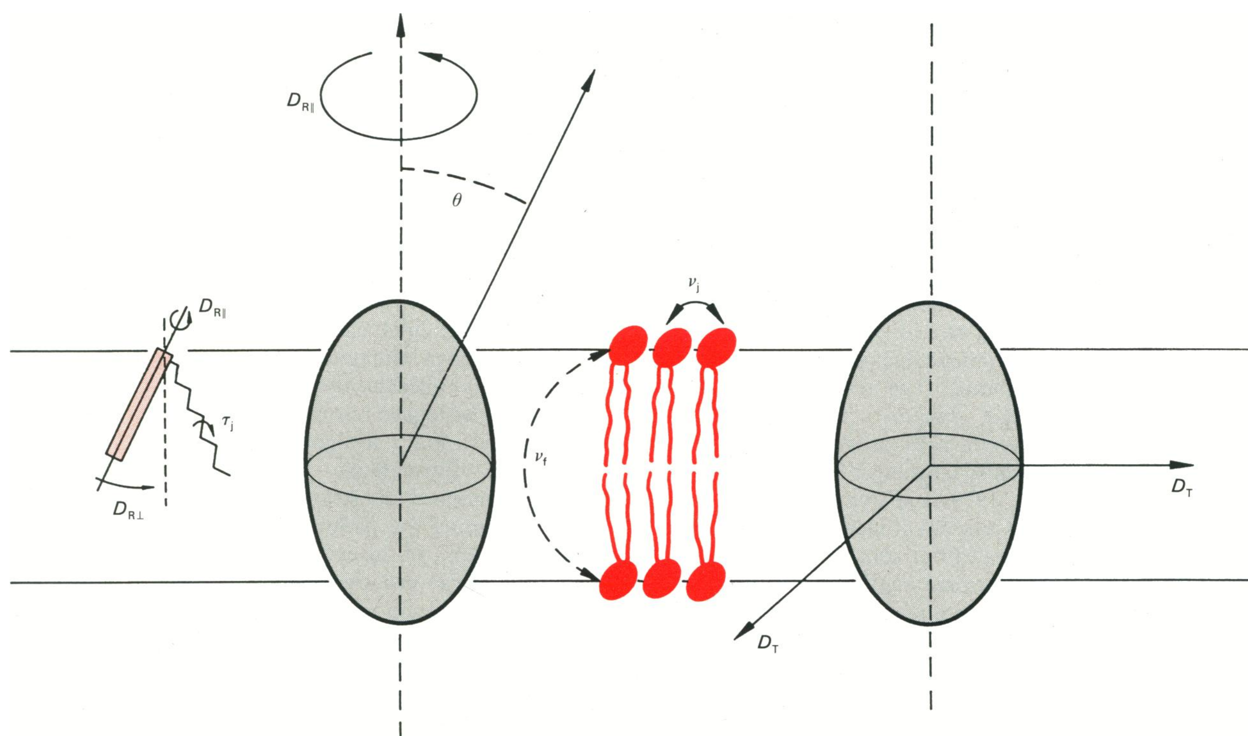


Fig. 1. Different modes of molecular motions in biological membranes

The lipid molecules rotate about their long axis with rotational diffusion coefficient $D_{R\parallel}$ and also perform limited rotation about perpendicular axes (within the plane of the membrane) with rotational diffusion coefficient $D_{R\perp}$. The lipid chains undergo *trans–gauche* isomerism about individual C–C bonds with a jump rate of τ_J^{-1}. The lipids also diffuse laterally within the plane of the membrane with hopping frequency, ν_j. The transbilayer diffusion rate, ν_f, for lipid molecules in pure lipid bilayers is very low, but protein-mediated translocation for specific lipids is faster in membranes. The integral protein molecules rotate about an axis perpendicular to the plane of the membrane with rotational diffusion coefficient $D_{R\parallel}$, and also may move laterally within the membrane plane with translational diffusion coefficient D_T (reproduced with permission from Marsh, 1988*a*).

Abbreviations used: lipids in Tables are abbreviated in accordance with IUPAC-IUB recommendations. Thus: Ptd, phosphatidyl; Cho, choline; Ins, inositol; Ser, serine; Etn, ethanolamine; Gro, glycerol; Myr, myristoyl; Ole, oleoyl.

Nuclear magnetic resonance (n.m.r.) spectroscopy is in principle possible with any nucleus having non-zero nuclear spin, depending on the sensitivity of the nucleus and its natural abundance. Examples of biological relevance include ^{1}H, ^{2}H, ^{13}C, ^{14}N, ^{15}N and ^{31}P. The hydrogen nucleus ^{1}H, for example, has magnetic spin quantum numbers of $\pm\frac{1}{2}$ which result in two distinct energy states when a magnetic field is applied. Nuclear magnetic resonance is observed when radio-frequency radiation with energy quantum matching the difference between these energy states is applied. It is clear that there are a vast number of hydrogen nuclei in a particular membrane protein or lipid assembly, many of which are in different chemical environments that in principle may yield distinct, conformationally sensitive n.m.r. signals. In contrast to the conditions for normal high-resolution n.m.r. where the molecules are tumbling rapidly, the molecular motions in membranes are slow. Anisotropic interactions are therefore not averaged and this results in 'broad line' n.m.r. spectra. Such spectra contain valuable orientational and conformational information and, through relaxation and line shape studies, also give dynamic information. The anisotropic features which have been most frequently exploited are quadrupolar splittings from isotopically labelled ^{2}H groups (Seelig, 1977; Seelig & Seelig, 1980; Griffin, 1981; Davis, 1979) and chemical shift anisotropy from ^{31}P in the phosphate of the lipid head group (Seelig, 1978) or from ^{13}C-labelled carbonyls in the backbone region (Blume *et al.*, 1982). Dipolar interactions are resolved only for magnetically dilute nuclei and hence, for ^{1}H n.m.r., are difficult to exploit. The general strategy has been to eliminate proton dipolar interactions through rapid tumbling in the case of small extrinsic proteins and sonicated lipid vesicles, or by solid state n.m.r. methods. High-resolution solid state n.m.r. methods are particularly powerful when studying isotopically enriched nuclei, e.g. ^{13}C. In addition, dynamic information can be obtained from the n.m.r. relaxation behaviour which depends on magnetic fields from the surrounding nuclei and electrons that fluctuate as a result of the molecular motion.

Electron spin resonance (e.s.r.) is a phenomenon similar to n.m.r. but which requires unpaired (i.e. paramagnetic) electrons. E.s.r. is thus extremely specific in its application, and many membranes give no intrinsic e.s.r. signals since they contain no unpaired electrons. The majority of e.s.r. studies on membranes involve attachment of stable, synthetic free radicals ('spin labels') at defined sites in the membrane. The resulting spectra are anisotropic and give similar dynamic, orientational and conformational information on the lipids as is obtained by broad line n.m.r. (Marsh, 1981), whilst labelling of the protein gives conformational (Coan *et al.*, 1979) and dynamic (Fajer *et al.*, 1989) information. An important class of membranes which give e.s.r. signals directly (i.e. without prior spin labelling) are those involved in redox reactions where paramagnetic transition metals or organic free radicals such as semiquinones participate.

It is the aim of this review to describe the different types of information obtained from application of magnetic resonance methods in the study of membranes. The review is intentionally not comprehensive in all aspects; the literature in this area is vast. In certain cases, we have preferred rather to select particular examples to illustrate the value of these powerful methods.

HIGH-RESOLUTION N.M.R.: MOLECULAR CONFORMATION

Historically it was first demonstrated that high-resolution ^{1}H, and later ^{13}C and ^{31}P, n.m.r. spectra can be obtained from small sonicated phospholipid vesicles. The high resolution spectra arise because dipole–dipole interactions and chemical shift anisotropies (for ^{31}P and ^{13}C), which would give rise to broad-line spectra, are averaged by the rapid isotropic vesicle rotation. These vesicles are composed of a single bilayer membrane, which is, however, highly curved. The lipid chain packing in small vesicles is therefore considerably strained compared with extended bilayer membranes, and for example the chain melting phase transition is considerably broadened. The sonicated vesicles constitute a convenient model system in which barrier properties, in particular the permeability of n.m.r. shift reagents and the inside-outside distribution of lipid species, can be studied, provided packing distortions are not critical.

Table 1. ^{31}P n.m.r. chemical shifts of phospholipids in potassium cholate or [Triton X-100], pH 8 (London & Feigenson, 1979)

Upfield shifts are positive, relative to external 86% H_3PO_4.

Lipid	Chemical shift (p.p.m.)
PtdCho	+0.65 [+0.9]
PtdIns	+0.40
Lyso PtdCho	+0.15
PtdSer	+0.12
PtdEtn	+0.00 [+0.25]
Sphingomyelin	+0.00
Ptd_2Gro	−0.31
PtdGro	−0.43
External 1 M-P_i, pH 7.0	−2.1
PtdH	−3.8

In general, the high resolution spectra from lipid vesicles resolve the chemical shift differences between the lipid headgroup, glycerol backbone and certain of the chain resonances (for ^{1}H and ^{13}C), but not the spin–spin splittings. To date, spin–spin splittings have been resolved for conventional n.m.r. conformational analysis only in very limited cases (Marsh & Watts, 1978). For ^{31}P, the chemical shifts are characteristic of the different lipid species and can be utilized in the quantitative analysis of the lipid composition of sonicated vesicles or detergent-solubilized membranes (see Table 1). In addition, ^{13}C chemical shifts provide an aid to conformational analysis, for example of the lipid chains (Batchelor *et al.*, 1972). ^{13}C T_1 relaxation time measurements also provide valuable information on segmental molecular dynamics (Lee *et al.*, 1976), benefitting both from the higher resolving power of ^{13}C n.m.r. relative to proton n.m.r. and a straightforward analysis of the relaxation mechanism. Biosynthetic incorporation of isotopic labels from ^{13}C-enriched substrates also offers a valuable method for investigating the pathways of lipid biosynthesis, via high-resolution n.m.r. of the extracted membrane lipids (Smith *et al.*, 1978). Nuclear Overhauser effects (^{31}P–[^{1}H]) have also been used to provide information regarding headgroup conformation in sonicated lipid systems (Yeagle *et al.*, 1976, 1977).

Recent developments with lipid systems include the use of two-dimensional n.m.r. methods to define Overhauser enhancements and hence proximity relationships (Xu & Cafiso, 1986), but the most significant developments are to be expected in the solid-state n.m.r. of unsonicated lipid dispersions. Magic-angle spinning has been used to eliminate residual dipolar interactions and obtain high-resolution ^{1}H n.m.r. spectra of unsonicated lipid dispersions and the myelin membrane (Oldfield *et al.*, 1987; Oldfield, 1988). Two-dimensional n.m.r. techniques have demonstrated the conformational distortion of the lipid molecules in sonicated systems, relative to the unsonicated lipid dispersions (Forbes *et al.*, 1988).

High-resolution spectra can be resolved for acylglycerols in cells (Mountford & Wright, 1988) and also even in whole body

n.m.r. The rapid rotation of the acylglycerol molecules in fat droplets gives rise to sharp n.m.r. resonances, as in conventional high-resolution spectroscopy. This aspect is especially interesting, since such signals are found predominantly in malignant cells, which may point to an abnormality in the lipid metabolism.

Membrane bound proteins do not give rise to high-resolution spectra using normal solution n.m.r. techniques, because the rotational motions are too slow to average the anisotropic interactions that cause line broadening. High-resolution spectra may be obtained from peripheral proteins in aqueous solution, good examples being the electron transport protein cytochrome *c* (Gao *et al.*, 1990) and the encephalitogenic myelin basic protein (Mendz *et al.*, 1982, 1983*a*,*b*, 1986). High resolution spectra may be obtained from surface-active peptides, or small integral proteins, and from peptides derived from integral proteins, either in organic solvents or detergent solution. In the case of ^{1}H-n.m.r., perdeuterated detergents (and solvents) are required.

One of the first determinations of the structure of a micelle-bound peptide using two-dimensional n.m.r. techniques was that of the hormone glucagon (Braun *et al.*, 1981). The structure of the bee venom peptide mellitin bound to detergent micelles has been determined by using two-dimensional n.m.r. techniques to give complete sequence-specific assignments and distance connectivities necessary for the distance geometry calculations (Inagaki *et al.*, 1989). The mellitin was found to form an α-helical rod, with a 120–160° kink at Thr-11 and Gly-12, which binds parallel to the lipid–water interface. The cytolytic peptide δ-haemolysin in micelles was found to have a central region which forms an extended helix with a pronounced amphipathic distribution of polar and nonpolar amino acid side chains (Lee *et al.*, 1987). Similar analyses have been carried out for mellitin (Bazzo *et al.*, 1988), δ-haemolysin (Tappin *et al.*, 1988) and the voltage-gated channel peptide alamethicin (Esposito *et al.*, 1987) in methanol solution. In the case of mellitin and δ-haemolysin, the structure was found to be similar to that bound to micelles.

A particularly interesting application of this approach is the determination of the solution structure of the S4 segment of the sodium channel protein (Mulvey *et al.*, 1989). This segment is highly conserved among different species, contains a positively charged residue at almost every third position in its sequence, and is therefore thought to confer the voltage sensitivity on the sodium channel. The peptide was found to form a predominantly α-helical conformation in trifluoroethanol/water (9:1), which is consistent with the 'sliding helix' model of channel activation, but with a largely unstructured *C*-terminal that may explain the lack of influence on gating charge of positively charged residue substitutions in this region of the protein.

High-resolution ^{1}H n.m.r. spectra have been obtained from some of the aromatic residues of bacteriorhodopsin in detergent (Mayo *et al.*, 1988). About nine out of the total 32 aromatic residues were found to be relatively mobile, and photochemically-induced dynamic nuclear polarization studies revealed that only one of the three tyrosines and one of the two tryptophan residues were exposed to solvent. Studies on the smaller Pf1 bacteriophage coat protein in detergent micelles allowed determination of the structure of a major part of the protein using two-dimensional ^{1}H and ^{15}N n.m.r. techniques (Schiksnis *et al.*, 1987). Sequence-specific assignments of the slowly exchanging amide protons (facilitated by specific ^{15}N labelling) made it possible to demonstrate that residues 30–40 of the hydrophobic mid-section of the protein have a helical secondary structure.

Proteins in intact membrane systems can be studied only by solid-state n.m.r. techniques. High-resolution n.m.r. spectra are obtained for magnetically dilute spin-$\frac{1}{2}$ nuclei (e.g. ^{13}C) in solids by a combination of magic angle spinning, cross polarization and high-power proton decoupling. Magic angle

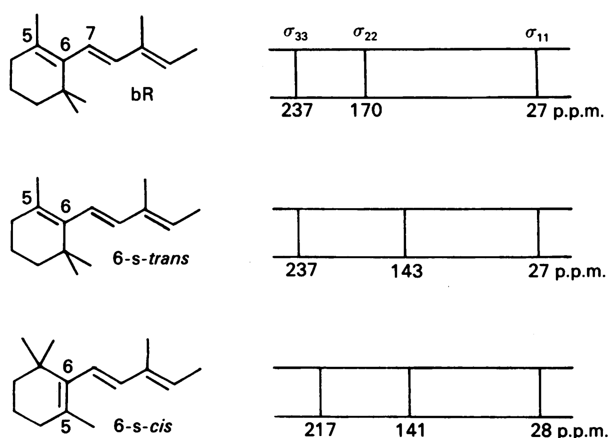


Fig. 2. Chemical shift tensor elements of the 5-^{13}C resonance of the retinal chromophore in bacteriorhodopsin (bR), compared with those of 6-s-*trans* and 6-s-*cis* retinoic acid

Isomerization of the C_6–C_7 single bond in the model compounds leads to a 20 p.p.m. shift in the σ_{33} tensor element. The shift in σ_{22} from $\approx$ 142 p.p.m. in the model compounds to 170 p.p.m. in bacteriorhodopsin is attributed to a negative protein charge near the 5-position in bacteriorhodopsin (Harbison *et al.*, 1985). In rhodopsin, in contrast to bacteriorhodopsin, the chromophore has unperturbed tensor elements characteristic of a 6-s-*cis* conformation (Mollevanger *et al.*, 1987; Smith *et al.*, 1987). Reproduced with permission from Smith & Griffin (1988); © 1988 Annual Reviews Inc.

spinning is used to remove the linebroadening due to chemical shift anisotropy and other similar anisotropic interactions; cross polarization gives rise to an increase in sensitivity by magnetization transfer from the proton spins; and heteronuclear decoupling removes the line broadening from the proton spins (for a review, see Smith & Griffin, 1988). Since the magnetically dilute nuclei (e.g. ^{13}C, ^{15}N) are frequently of low natural abundance, isotopic enrichment can also be used as an aid to assignment and to give site-specific probes.

Both structurally and functionally significant information has been obtained from the high-resolution solid-state n.m.r. of specifically ^{13}C-labelled retinals reconstituted in bacteriorhodopsin membranes (reviewed in Lugtenberg *et al.*, 1988). From the cross polarization/magic angle spinning spectra obtained at intermediate spinning rates, it was possible to determine the principal elements of the chemical shift anisotropy tensor, hence yielding more detailed structural information than could be determined from the isotropic chemical shifts alone (cf. Fig. 2). In this way it was possible to demonstrate that the two dark-adapted forms of bacteriorhodopsin, bR_{568} and bR_{548}, contain the chromophore in the all-*trans* and 13-*cis* forms, respectively (Harbison *et al.*, 1984*a*,*b*), that the C = N linkage of retinal to Lys-216 of the protein is in the *anti* conformation in bR_{568} and in the *syn* conformation in bR_{548} (Harbison *et al.*, 1984*b*), and that in both forms the C_6–C_7 bond of the chromophore is in the s-*trans* conformation (Harbison *et al.*, 1985; see Fig. 2). Cross polarization/magic angle spinning spectra from the M_{412} photointermediate of bacteriorhodopsin, which was kinetically trapped at low temperature, have demonstrated that the retinal-lysine Schiff base is unprotonated at this stage of the photocycle (Smith *et al.*, 1989). Further solid-state n.m.r. experiments on specifically ^{13}C-enriched retinals reconstituted in the visual receptor rhodopsin have shown that the 11-*cis* chromophore has the 6-s-*cis* conformation (cf. Fig. 2) and that the Schiff base linkage is *anti* (Smith *et al.*, 1987; Mollevanger *et al.*, 1987). One of these studies has also indicated that a negative charge on

rhodopsin is situated in the vicinity of the C_{12}–C_{13} region of the chromophore (Mollevanger *et al.*, 1987), and this has been implicated with the red shift in the visible spectrum of the opsin-chromophore.

BROADLINE ^{31}P N.M.R.: LIPID POLYMORPHISM

The ^{31}P-n.m.r. chemical shift anisotropy is partially averaged by molecular motion within the phospholipid headgroup and the extent of averaging can be used to aid conformational studies (Seelig, 1978; Smith & Ekiel, 1984). The most useful information which is normally obtained, however, comes from the additional motional averaging arising from the translational diffusion of the lipid molecules around the curved surfaces in non-lamellar lipid phases (Cullis & De Kruijff, 1979). Non-bilayer lipid phases may be involved in biologically important processes such as membrane biogenesis and fusion, and the import of nascent proteins into membranes. Recently, correlations have been established between the tendency of different lipids to induce spontaneous membrane curvature (and hence the potentiality to form non-lamellar phases) and the functional properties of various reconstituted membranes (Hui & Sen, 1989). These latter include: lipid hydrolysis by phospholipase A_2, phospholipid transfer between vesicles, passive permeability, incorporation of integral proteins into lipid membranes, and Ca^{2+}-ATPase pump activity (cf. also Navarro *et al.*, 1984). Cubic lipid phases have also been suggested to be involved in fat digestion (Mariani *et al.*, 1988; Lindblom & Rilfors, 1989).

The use of ^{31}P-n.m.r. in lipid phase identification is illustrated in Fig. 3, which demonstrates the polymorphism of lysophosphatidylcholine dispersed in polyethylene glycol solution (King & Marsh, 1989), the latter being a potent inducer of cell fusion. The powder patterns of the ^{31}P-n.m.r. spectra directly reflect the symmetry of the corresponding lipid phases. In the lamellar phase, L^i_β, the spectrum corresponds to axial symmetry and has a negative chemical shift anisotropy. In the hexagonal phase, H_I, the spectrum also indicates axial symmetry but has a chemical shift anisotropy, $\Delta\sigma$, which is of the opposite sign and less than half the size of that for the lamellar phase. This additional averaging arises from the translational diffusion of the lipid molecules about the hexagonal cylinder axis. In the cubic phase, Q, the spectral anisotropy is completely averaged. A single isotropic n.m.r. resonance is observed, corresponding to the higher symmetry of the phase. This is differentiated from the isotropic spectrum in the micellar phase, M, by the greater linewidth. In the latter case, true isotropic averaging arises from the rapid rotation of the small micelles, giving rise to a conventional high-resolution spectrum. A rich range of polymorphism has been found for cardiolipin analogues with differing numbers of fatty acyl chains (Powell & Marsh, 1985). Dilysocardiolipin with two chains disperses as micelles, whereas monolysocardiolipin and cardiolipin, which bear three and four chains per headgroup respectively, form bilayers, and cardiolipin with a fifth chain acylated on the central hydroxyl group forms solely an inverted hexagonal phase in water.

A particularly interesting application of the method is in the investigation of the effects of membrane proteins on the lipid polymorphic phase behaviour. Cytochrome *c* has been shown to be capable of inducing inverted hexagonal phases in bilayers of cardiolipin (De Kruijff & Cullis, 1980). This opens up the possibility that local non-lamellar lipid regions may be created in biological membranes by the action of extrinsic proteins. Integral proteins have been shown to stabilize the lamellar phase in lipids which otherwise form the H_{II} phase (Taraschi *et al.*, 1982, 1983; Rietveld *et al.*, 1987; Powell *et al.*, 1990). This stabilizing effect also extends to the natural lipid composition of biological

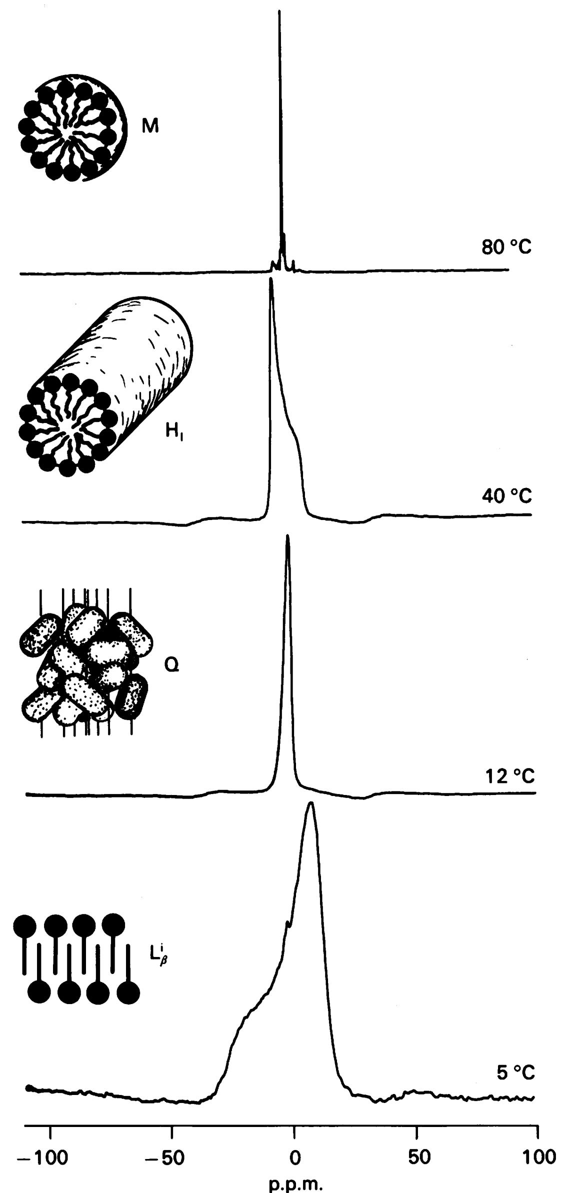


Fig. 3. Proton-dipolar decoupled 109 MHz ^{31}P n.m.r. spectra of palmitoyl lysophosphatidylcholine in 23 wt% aqueous polyethylene glycol

Spectra are recorded at increasing temperatures as indicated. Representations of the lipid phases are given on the left of the Figure: L^i_β, interdigitated lamellar gel; Q, fluid cubic (space group Pm3n); H_I, normal hexagonal; M, normal micellar. Reproduced with permission from King & Marsh (1989); © 1989 American Chemical Society.

membranes (De Grip *et al.*, 1979; Mollevanger & De Grip, 1984; Marsh & Esmann, 1991).

^{31}P n.m.r. measurements on oriented lipid membranes in the gel phase have been used to determine the orientation of the lipid phosphate group from the anisotropy in the chemical shift. The plane containing the two non-esterified oxygens is inclined at an angle of approximately 50° to the bilayer normal and the vector joining these two oxygens is oriented parallel to the bilayer surface (Griffin *et al.*, 1978). This orientation is essentially the same as found in the single-crystal X-ray structures of phospholipids (Hitchcock *et al.*, 1974; Pearson & Pascher, 1979). In fluid phase membranes, the chemical shift anisotropy is further reduced by the angular fluctuations of the phospholipid molecule. The chemical shift anisotropy of the different

phospholipids has been found to range from −55 p.p.m. for dimyristoyl phosphatidylserine (Browning & Seelig, 1980) to −32 p.p.m. for diphosphatidylglycerol (cardiolipin) (Powell & Marsh, 1985). In addition, lysolipids are found to have yet smaller values of the chemical shift anisotropy, indicating a somewhat different conformation of the headgroup from diacyl phospholipids (Powell & Marsh, 1985; Van Echteld *et al.*, 1981). The differences in chemical shift anisotropy can be used in favourable circumstances to identify phospholipids in mixed membranes, a good example being the investigation of phosphatidylcholine ($\Delta\sigma = -47$ p.p.m.)/phosphatidylethanolamine ($\Delta\sigma = -44$ p.p.m.) mixtures (Gawrisch *et al.*, 1977).

BROAD-LINE ^{2}H-N.M.R.: CHAIN CONFORMATION AND DYNAMICS; LIPID HEADGROUPS; PROTEIN SIDE CHAINS

The fluidity that is necessary for many membrane functions has its origin principally in the rotational isomerism of the lipid chains. This segmental rotational mobility and that of the other membrane components can be studied in detail by ^{2}H-n.m.r. of labelled molecular segments. The quadrupole splitting in the ^{2}H-n.m.r. spectrum depends on the angular orientation of the labelled group and the extent to which this is averaged by molecular motion (see, e.g., Seelig, 1977; Seelig & Seelig, 1980). The conformation, molecular ordering within the membrane, and the rates of segmental molecular rotation may therefore be studied with suitably ^{2}H-labelled membrane components. The techniques used are essentially those of solid state n.m.r., particularly quadrupolar echo spectroscopy, and have been reviewed by Griffin (1981) and Davis (1979).

The two principal features which have emerged from ^{2}H-n.m.r. studies of labelled phospholipid chains in membranes are the inequivalence of the *sn*-1 and *sn*-2 chains of glycerolipids and the chain flexibility gradient profile. The chain configuration about the glycerol backbone in fluid lipid bilayers has been shown to be similar to that in the crystal structure of phosphatidylcholines and phosphatidylethanolamines (Pearson & Pascher, 1979; Hitchcock *et al.*, 1974). The *sn*-1 chain is extended in the *trans* conformation from the glycerol backbone, whereas the *sn*-2 chain is bent at the C-2 position, in order to lie parallel with the *sn*-1 chain (Seelig & Seelig, 1975). This feature is preserved over a wide range of phospholipids (Seelig & Browning, 1978), and may be the conformational feature which is recognized in the chain specificity of phospholipase A_2. Results with ethanolamine plasmalogen, however, have shown that the conformation of the *sn*-2 chain in this lipid is different from that of diacyl glycerophospholipids (Malthaner *et al.*, 1987).

The profile of segmental order parameters with chain segment position has revealed that the chain order is relatively constant in the part of the chain closer to the lipid headgroups, but then drops off rapidly on approaching the terminal methyl ends of the chains (Seelig & Seelig, 1974). The chain order parameter is defined by:

$$S_n = \tfrac{1}{2}(3\langle \cos^2\theta_n \rangle - 1) \qquad (1)$$

where θ_n is the instantaneous angular orientation of the *n*th chain segment relative to the membrane normal, and the angular brackets indicate an average taken over the entire chain motion, i.e. both long axis motions and *trans–gauche* isomerism; cf. Fig. 1. The order parameter results define the basic features of the lipid chain packing: a high degree of co-operativity in the upper part of the chain and more independent motion allowed by the free volume created by differential shortening at the chain ends. Similar effects are observed in natural membranes as in lipid model membranes (Stockton *et al.*, 1977). Increasing temperature decreases the order parameters and tends to diminish the extent of the plateau region of near-constant order parameter (Seelig & Seelig, 1974), whereas chain unsaturation leads to a general disordering but tends to increase the degree of local order in the vicinity of the double bond (Seelig & Waespe-Sarcevic, 1978; Rance *et al.*, 1980). The lipid polar headgroup has only minor effects on the lipid chain order (Marsh *et al.*, 1983), whereas cholesterol has a very pronounced ordering effect on the lipid chains (Oldfield *et al.*, 1978).

For molecules with ring systems that impose fixed conformational constraints, it is possible to determine the full ordering tensor and hence to distinguish molecular orientation from segmental fluctuations in conformation. This has been done by systematic ^{2}H-substitution in cholesterol (Dufourc *et al.*, 1984), in lipid chains bearing a cyclopropane ring (Dufourc *et al.*, 1983), and in glycolipid headgroups (Jarrell *et al.*, 1986, 1987*a,b*; Renou *et al.*, 1989). In the latter case, it was found that the headgroup of lactosyl diacylglycerol was extended away from the membrane surface into the aqueous phase, and that the order parameters of the glucose and galactose segments were identical, indicating that the headgroup fluctuates about the membrane normal as a rigid unit (Renou *et al.*, 1989).

The quadrupole splittings of phospholipids ^{2}H-labelled in the headgroup also contain conformational information, but insufficient constraints are available to provide a unique analysis (Seelig *et al.*, 1977; Skarjune & Oldfield, 1980). The data can, however, be fit consistently by assuming that the polar headgroups of both phosphatidylcholine and phosphatidylethanolamine are oriented parallel to the membrane surface, as in their crystal structure (Hitchcock *et al.*, 1974; Pearson & Pascher, 1979), and undergo transitions between equivalent conformations. More recently, it has been demonstrated that the orientation and conformation of the headgroup of phosphatidylcholine (and phosphatidylethanolamine), as recorded by the ^{2}H-quadrupole splittings, are exquisitely sensitive to the electrostatic charge density at the membrane surface (Scherer & Seelig, 1989). Positively charged amphiphiles are found to move the N^+ end of the headgroup dipole out into the aqueous phase, whereas negatively charged amphiphiles have the opposite effect, forcing the N^+ end in towards the membrane interior. Similar effects are also induced by binding of ions (Altenbach & Seelig, 1984; MacDonald & Seelig, 1987) and charged peptides or proteins (Sixl & Watts, 1985; Roux *et al.*, 1989; Kuchinka & Seelig, 1989; Dempsey *et al.*, 1989; Sixl *et al.*, 1984).

Integral proteins have been found to have relatively small effects on the lipid chain ordering (for reviews see Seelig *et al.*, 1982; Bloom & Smith, 1985; Marsh & Watts, 1988). Single-component ^{2}H-n.m.r. spectra are observed corresponding to fast exchange between all lipid environments on the characteristic timescale of ^{2}H-n.m.r., since this is not as well matched to the lipid exchange rate at the protein interface as is that for spin label e.s.r. The effects of lipid–protein interactions are therefore less directly manifested than in the latter technique which is discussed in the following section. Reconstitution studies with rhodopsin have suggested that, whereas the mean chain order parameter remains relatively unchanged, the spread in order parameter distribution is increased, by virtue of the heterogeneity of environments for the lipid chains in the presence of protein (Bienvenue *et al.*, 1982). Studies on reconstituted myelin proteolipid protein have indicated that neither the segmental chain order parameter nor the orientational order parameter of the lipid chain axis are appreciably affected by the integral protein (Meier *et al.*, 1987). The principal effect of the lipid–protein interaction is upon the chain mobility: detailed studies of the myelin proteolipid system have indicated a progressive slowing down of all components of the lipid chain

motions, by up to a factor of 10 at very high protein contents (Meier *et al.*, 1987).

Biosynthetic incorporation of ^{2}H-labelled amino acids allows the study of the dynamics of membrane proteins (Kinsey *et al.*, 1981; Keniry *et al.*, 1984*a*). For bacteriorhodopsin in the purple membrane of *H. halobium* such studies have been used to assess which amino acid side chains have appreciable mobility and to determine the distribution of mobile resonances between the surface and interior groups (Keniry *et al.*, 1984*b*; Herzfeld *et al.*, 1987). In the second reference quoted it was claimed that few if any aromatic residues in bacteriorhodopsin are highly mobile (rotational rates are $< 10^5\ s^{-1}$) and that the previous interpretations of the protein dynamics should be reviewed. Biosynthetically incorporated ^{2}H- and ^{15}N-labelled amino acids have been used to study both the backbone and side chain dynamics of the fd bacteriophage coat protein in phospholipid bilayers (Leo *et al.*, 1987; Bogusky *et al.*, 1988). Residues 6–42 were found to have immobile backbone sites, whereas the four *N*-terminal residues are highly mobile and the last seven *C*-terminal residues display a mobility gradient, with the two terminal residues being highly mobile. The side chains of many of the residues in the rigid backbone region undergo rotational flips, with the exception of the single tryptophan which is completely immobile on the timescale of the measurement.

Solid state n.m.r. can also be used for the analysis of protein conformation by using biosynthetically incorporated ^{13}C- and ^{15}N-labelled amino acids. Combination of a variety of anisotropic interactions, particularly heteronuclear dipolar, allows the determination of the orientation of the peptide plane and hence of the protein backbone conformation (reviewed by Opella *et al.*, 1987).

SPIN-LABEL E.S.R.: LIPID–PROTEIN INTERACTIONS

Lipid–protein interactions are important determinants of cellular membrane structure and function. They are responsible not only for the sealing of integral proteins into the membrane, but also provide the interface with the fluid lipid membrane environment which is necessary for the function of many membrane enzymes and transport proteins. Specific lipid–protein interactions are also important in certain systems. The activity of various mitochondrial redox enzymes, particularly cytochrome oxidase, is enhanced by cardiolipin (Abramovitch *et al.*, 1990); local anaesthetics which act as non-competitive blockers of the acetylcholine receptor bind at the lipid–protein interface (Horváth *et al.*, 1990*c*); and free fatty acids are found to be either activators or inhibitors of particular membrane enzymes (see e.g. Marsh, 1987). A short review of recent e.s.r. results on lipid–protein interactions with both integral and peripheral proteins has been given by Marsh (1990).

The e.s.r. spectra of phospholipid molecules bearing a spin label on the acyl chain are optimally sensitive to the rotational mobility of the lipid chains in membranes (for a detailed analysis, see Lange *et al.*, 1985; Moser *et al.*, 1989). The motional sensitivity arises from the rotational modulation of the spectral anisotropies (hyperfine and *g*-tensors) and is therefore limited to motions in the time range 10^{-11} to 10^{-8} s. Jost *et al.* (1973) were the first to demonstrate that the boundary layer of lipids surrounding integral proteins has a restricted mobility relative to that of the bulk bilayer lipids. This was evidenced by the resolution of two-component e.s.r. spectra from spin-labelled lipids which were exchanging only slowly relative to the 10^{-8} s e.s.r. motional limit. Quantification of the relative intensities of the two components, using spectral subtraction, yields information on the stoichiometry and specificity of the lipid-protein interaction (see e.g. Marsh, 1983, 1985). The appropriate equation for lipid-protein association (Brotherus *et al.*, 1981; Knowles *et al.*, 1979) is:

$$(1-f)/f = (n_t/N_1 - 1)/K_r \qquad (2)$$

where f is the fraction of motionally restricted spin-labelled lipid component in the e.s.r. spectrum, n_t is the total lipid/protein ratio in the sample, N_1 is the number of first-shell (or boundary layer) lipid sites on the protein, and K_r is the association constant of the spin-labelled lipid relative to the unlabelled background host lipid. The stoichiometry of the interaction, specified by N_1, correlates well with the size of the intramembranous surface of the proteins involved (see Table 2). The lipid specificity pattern, given by the relative values of K_r, is complex and is characteristic of the particular membrane protein. The highest selectivities are observed for negatively charged phospholipids, but the specificities for lipids with the same formal charge are not identical. In general, changing the charge on the lipid by pH titration has a greater effect on the selectivity than screening the electrostatic interactions by high ionic strength. These results have been reviewed by Marsh (1983, 1985, 1987, 1988*b*). Interestingly, monogalactosyl diacylglycerol in thylakoid membranes appears to take the part played normally by phosphatidylcholine in mammalian membranes, where the latter is the majority non-specific lipid. Lipid specificity is found, however, for phosphatidylglycerol in thylakoid membranes, which is unusual in non-plant systems (Li *et al.*, 1989*a*).

In addition to the structural and thermodynamic parameters of the lipid–protein interaction, simulation of the two-component e.s.r. spectra from lipid–protein systems (using the exchange-coupled Bloch equations) yields information on the dynamics of the lipid-protein interaction (Davoust & Devaux, 1982; Marsh, 1985; Horváth *et al.*, 1988*a*). The conjugate variables obtained from the simulations are the first-order rate constant for exchange from the motionally restricted lipid component, τ_b^{-1}, and the fraction of motionally restricted lipid, f. The on-rate for exchange from the fluid lipid component is simply related to the off-rate, τ_b^{-1}, by the condition for mass balance: $(1-f)\tau_f^{-1} = f\tau_b^{-1}$. The values for the off-rate constant obtained from simulation are given in Table 3. These off-rates are in the region of $10^7\ s^{-1}$, with that for the M-13 coat protein being approximately twice as slow as for the other proteins. The exchange rate at the lipid–protein interface therefore is much faster than the frequencies characteristic of the quadrupole splittings in ^{2}H-n.m.r. spectroscopy (maximally $\approx 2 \times 10^6\ s^{-1}$, for rigid systems). This explains why single-component ^{2}H-n.m.r. spectra are observed for deuterium-labelled lipids in lipid–protein systems, as discussed in the previous section.

The values of the off-rate constant in Table 3 are of the same order, but significantly slower, than the intrinsic lipid–lipid exchange rates arising from lateral diffusion in fluid lipid bilayers (see below). The reason for this could be partly a steric hindrance of the diffusive motion of the lipid by the protein, but may also indicate that the hydrophobic part of the lipid–protein interaction is energetically somewhat more favourable than that for the lipid–lipid interaction.

The energetics of the selectivity of the lipid-protein interaction $[\Delta G^\circ \approx -RT\ln(K_r)]$ are also expected to be reflected in the exchange dynamics. Combination of the condition for mass balance with eqn. (2) yields the following relationship between the lipid exchange rate and relative association constant (Horváth *et al.*, 1988*a*):

$$\tau_b^{-1}/\tau_f^{-1} = (n_t/N_1 - 1)/K_r \qquad (3)$$

If it is assumed that in eqn. (3) the on-rate, τ_f^{-1}, is the same for all lipids (i.e. is diffusion-controlled), then the ratios of the off-rate constants for different lipids would be expected to be in the

Table 2. Stoichiometries of the motionally restricted lipid component, N_1^{exp}, determined by e.s.r. in various lipid-protein systems and estimates of the number of first-shell lipids, N_1^{calc}, that can be accommodated around the integral proteins

Protein/membrane	$10^{-3} \times M_r$	N_1^{exp} (mol/mol)	$N_1^{exp}/\sqrt{M_r}$	N_1^{calc} (mol/mol)	Reference¶
Na^+,K^+-ATPase/ Ole_2PtdCho	314	63±3	0.112 ±0.005	(≈ 57–72)	1
Na^+,K^+-ATPase/ shark rectal gland	294	66±6	0.122 ±0.011	(≈ 57–72)	2
Cytochrome oxidase/ Myr_2PtdCho	165	45±4	0.110 ±0.011	40–45	3
Acetylcholine receptor/ Ole_2PtdCho	250	40±7	0.080 ±0.014	43	4
Ca^{2+}-ATPase/ egg PtdCho	115	22±2	0.065 ±0.006	(≈ 23–27)*	5
Sarcoplasmic reticulum Ca^{2+}-ATPase	115	24±5	0.071 ±0.015	(≈ 23–27)*	6
Rhodopsin/Myr_2PtdCho	39	23±3	0.116 ±0.015	(24±2)	7
Bovine rod outer segment disc/rhodopsin	39	25±3	0.125 ±0.016	(24±2)	8
Frog rod outer segment disc/rhodopsin	39	22±2	0.114 ±0.010	24	9
Myelin proteolipid/ Myr_2PtdCho	25	10±2	0.063 ±0.013	(≈ 10–12)†	10
ADP–ATP carrier/ egg PtdCho	65.6‡	50	0.195	31–34‡	11
M-13 coat protein/ Myr_2PtdCho	5.2	4	0.055	5§	12

* Calculated assuming a dimer, with monomer radius 2 nm (ref. 13).
† Calculated assuming a hexamer.
‡ For dimer.
§ Calculated for linear polymer or tetramer.
¶ References: 1, Brotherus *et al.* (1981); 2, Esmann *et al.* (1985, 1988); 3, Knowles *et al.* (1979); 4, Ellena *et al.* (1983); 5, Silvius *et al.* (1984); 6, Thomas *et al.* (1982); 7, Ryba *et al.* (1987); 8, Watts *et al.* (1979); 9, Pates *et al.* (1985), Marsh *et al.* (1982); 10, Brophy *et al.* (1984); 11, Horváth *et al.* (1990*a*); 12, Wolfs *et al.* (1989); 13, Marsh & Watts (1982).

inverse ratio of the relative association constants. This is found to be the case both for the myelin proteolipid protein (Horváth *et al.*, 1988*a,b*) and for the M-13 coat protein (Wolfs *et al.*, 1989). Lipids such as phosphatidic acid, stearic acid or phosphatidylserine, which display a selectivity for the protein ($K_r > 1$), are found to have off-rates which are slower than those given for phosphatidylcholine in Table 3. In addition, when the lipid selectivity is reduced either by pH titration or by electrostatic screening at high ionic strength, the on-rates remain constant, indicating that they are diffusion-controlled, whereas the off-rates increase, reflecting exactly the decreased specificity(Horváth *et al.*, 1988*b*). This method has been used recently to identify a non-exchanging, or only slowly exchanging, population of diphosphatidylglycerol (cardiolipin) that is tightly associated with the mitochondrial ADP–ATP carrier (Horváth *et al.*, 1990*a*).

Table 3. Lipid exchange off-rate constants, τ_b^{-1}, (and activation energies, E_a) for various integral membrane proteins reconstituted in phosphatidylcholine bilayers, obtained by simulation of the e.s.r. spectrum of spin-labelled phosphatidylcholine, using the exchange-coupled Bloch equations

Protein	T (°C)	τ_b^{-1} (s^{-1})	E_a (kJ/mol)	Reference*
Myelin proteolipid protein/Myr_2PtdCho	30	1.6×10^7	20	1
Myelin DM-20 protein/ Myr_2PtdCho	30	1.5×10^7	–	2
Rhodopsin/Myr_2PtdCho	30	1.6×10^7	20	3
M-13 coat protein/ Myr_2PtdCho	30	5.3×10^6	–	4
ADP–ATP carrier/ egg PtdCho	10	1.4×10^7	–	5
Ca^{2+}-ATPase/ Ole_2PtdCho	37	$(1–2) \times 10^7$	–	6

* References: 1, Horváth *et al.* (1988*a,b*); 2, Horváth *et al.* (1990*d*); 3, Ryba *et al.* (1987); 4, Wolfs *et al.* (1989); 5, Horváth *et al.* (1990*a*); 6, East *et al.* (1985).

SATURATION-TRANSFER E.S.R.: PROTEIN ROTATION

The oligomeric state of membrane proteins can be critical for the function. For example, a dimeric model has been proposed for the transport mechanism of the mitochondrial ADP–ATP carrier (Klingenberg, 1981). Certain aspects of the kinetics of the Na,K-ATPase can also best be explained in terms of a dimeric model (Plesner *et al.*, 1981). The aggregation state of multienzyme complexes, e.g. the mitochondrial electron transport chain, may also be important for function. Associations between intrinsic and extrinsic proteins may be of either functional or structural significance, particularly in the case of the interaction of cytoskeletal elements with the membrane. Such associations and interactions can be sensitively detected by measurements of the rotational mobilities of the component proteins.

The rotational diffusion of covalently spin-labelled integral membrane proteins can be studied using saturation transfer e.s.r. (Thomas *et al.*, 1976). The timescale of this method is determined by the spin-lattice relaxation times, T_1, which for spin labels in these systems lie in the microsecond to tens of microseconds range. This corresponds exactly to the time regime for the

rotational mobility of integral proteins in membranes. Effective correlation times, τ_R^{eff}, are deduced from standard spectra of spin-labelled haemoglobin rotating isotropically in glycerol solutions of varying viscosity. Calibrations in readily accessible form are given by Horváth & Marsh (1988). Further details can be found in Marsh (1981), and experimental considerations are reviewed in Marsh (1989) and Marsh & Horváth (1989).

The rotational diffusion of proteins in membranes is highly anisotropic, being essentially restricted to rotation about the membrane normal (see Fig. 1). Spectral simulations for anisotropic diffusion have been performed by Robinson & Dalton (1980). It was found that the effective correlation times deduced experimentally by comparison with isotropic model systems are related to the orientation, θ, of the spin label z-axis with respect to the rotation axis by:

$$\tau_R^{eff}(\pm 1) = 2\tau_{R\parallel}/\sin^2\theta \quad (4)$$

where $\tau_{R\parallel}$ ($= 1/6D_{R\parallel}$; cf. Fig. 1) is the rotational correlation time about the protein axis. In the absence of detailed knowledge of the orientation of the spin label with respect to the protein, the maximum value of the rotational correlation time, corresponding to $\theta = 90°$, is therefore given by: $\tau_{R\parallel} = \tau_R^{eff}/2$. Experimental values obtained for a variety of different spin-labelled integral proteins are given in Table 4. The values display a very clear dependence on protein size. The Na,K-ATPase and cytochrome oxidase are large integral proteins of monomer molecular masses 147 and 165 kDa, respectively, whereas the ADP–ATP carrier is a small hydrophobic protein with molecular weight 39 kDa.

The rotational correlation time can be related to the protein size using hydrodynamic theory. For a right cylindrical protein of circular cross-section, embedded in a membrane of viscosity η, the diffusion coefficient is given by (Saffman, 1976):

$$\tau_{R_\parallel} = 2\pi\eta a^2 h/(3kT) \quad (5)$$

where $h \approx 4.5$ nm is the height of the membrane-spanning region of the cylinder and a is the intramembranous radius of the cylinder. Appropriate values for the membrane viscosity are η = 2–5 P (Cherry & Godfrey, 1981), which were obtained by calibration with bacteriorhodopsin whose intramembranous cross-sectional dimensions are known. The data given in Table 4 are found to be consistent with a diprotomer or higher oligomer for the Na,K-ATPase (Esmann *et al.*, 1987, 1989), with a dimer of approximate cross-sectional dimensions 10 × 6 nm for cytochrome oxidase (Fajer *et al.*, 1989), and with a dimer of size approximately 4.6 × 4 nm for the ADP–ATP carrier (Horváth *et al.*, 1989).

Protein–protein interactions have been studied for reconstituted cytochrome oxidase by measuring the rotational diffusion rate as a function of protein packing density (Fajer *et al.*, 1989). Purely transient, random associations were observed, in agreement with current models for the electron transport process in mitochondria (Hackenbrock *et al.*, 1986). Interactions between erythrocyte band 3 and cytoskeletal proteins have been studied via the increase in rotational correlation time of the band 3 protein (Sakaki *et al.*, 1982). Rotational diffusion measurements on rhodopsin reconstituted in lipids of different chainlength have been used to establish the optimum chainlength required to incorporate the protein in the membrane without lateral segregation (Kusumi & Hyde, 1982). Factors governing the rotational mobility of the Ca^{2+}-ATPase have been extensively studied in an attempt to correlate protein mobility with enzymatic activity (Bigelow *et al.*, 1986; Bigelow & Thomas, 1987; Squier *et al.*, 1988*a*,*b*; Squier & Thomas, 1988). It was concluded that protein rotational mobility is most probably essential for the rate limiting step in active calcium transport. Saturation-transfer e.s.r. has been used to study the restriction in rotational mobility of the lipids associated with the PS2 photoreaction centre (Li *et al.*, 1989*b*). Such studies are relevant to the rate-limiting step in oxygen evolution, since they concern the accessibility of the lipid-soluble plastoquinone to the reaction centre. Measurements on gel phase lipid bilayers as model systems (Marsh, 1980) provide a valuable guide in such studies, as well as being of fundamental importance in the analysis of anisotropic rotation.

Table 4. Maximal values of the rotational correlation times, $\tau_{R\parallel}$, of spin-labelled integral proteins, deduced from saturation transfer e.s.r. spectra, using isotropic calibrations and assuming $\theta = 90°$ in eqn. (4)

The rotational correlation time is defined by: $\tau_{R\parallel} = \frac{1}{6}D_{R\parallel}$, where $D_{R\parallel}$ is the rotational diffusion coefficient; cf. Fig. 1.

Protein	T (°C)	$\tau_{R\parallel}$ (μs)	Reference*
Na,K-ATPase	20	25	1
ADP–ATP carrier	30	2	2
Cytochrome oxidase/ Myr_2PtdCho	30	25	3
Ca^{2+}-ATPase	4	25–40	4
Band 3/Myr_2PtdCho	37	4.3†	5
Rhodopsin/Myr_2PtdCho	30	6	6
M-13 coat protein/ Myr_2PtdGro	31	10	7

* References: 1, Esmann *et al.* (1987, 1989); 2, Horváth *et al.* (1989); 3, Fajer *et al.* (1989); 4, Horváth *et al.* (1990*b*); 5, Sakaki *et al.* (1982); 6, Kusumi & Hyde (1982); 7, De Jongh *et al.* (1990).
† Calculated with $\theta = 20°$.

LIPID LATERAL DIFFUSION

The concentration-dependent spin–spin interactions between spin-labelled lipid molecules may be used to measure translational diffusion rates, or the degree of lipid segregation in systems with limited diffusion. Historically, this method provided the first demonstration of rapid lateral diffusion of lipid molecules in membranes (Träuble & Sackmann, 1972; Devaux *et al.*, 1973). The spin label concentration range used depends on whether the spin-labelled molecule is an integral part of the system, as when studying lipid segregation, or whether probe amounts, as for lateral diffusion measurements, are required. The translational diffusion coefficients of various spin-labelled lipid derivatives in fluid lipid membranes are given in Table 5. These values are rather similar for all the different phospholipid species and imply rather fast lipid hopping rates (cf. Fig. 1): $\nu_j \approx 4D_T/\langle x^2\rangle \approx$

Table 5. Lateral diffusion coefficients, D_T, of different spin-labelled lipids in dimyristoyl phosphatidylcholine bilayers at 30 °C (Sachse *et al.*, 1987; Sachse, 1986)

The lipid jump frequency (cf. Fig. 1) is given by: $\nu_j \approx 4D_T/\langle x^2\rangle$, where $\langle x^2\rangle$ is the mean square displacement between adjacent lipid sites (Marsh, 1988).

Lipid	$10^7 \times D_T$ ($cm^2 \cdot s^{-1}$)
PtdCho	1.2
PtdH	1.0
Stearic acid	1.6
Ganglioside G_{M1}	1.0
Sphingomyelin	1.3

$(5–10) \times 10^7\ s^{-1}$. Typically, for instance, a lipid molecule will diffuse distances of the order of microns in a second. Similar methods may be used to study the segregation of different lipids within the membrane. Examples of calcium-induced phase separation of anionic spin-labelled phospholipids have been given by Galla & Sackmann (1975).

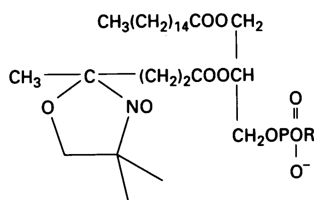
N.m.r. methods are available for measuring long-range lipid lateral diffusion using a field gradient. This method is applicable either to isotropic cubic lipid phases, or lipid bilayers oriented at the magic angle in order to obtain high resolution n.m.r. spectra (Lindblom & Wennerström, 1977; Rilfors *et al.*, 1986). Comparison between long-range (n.m.r.) and short-range (e.s.r.) lateral diffusion is particularly useful in structural investigations of cubic lipid phases, since it may be used to distinguish between continuous three-dimensional structures and localized structures of cubically arranged micelles (Lindblom & Rilfors, 1989).

SPIN LABEL PROBE METHODS

Transmembrane lipid asymmetry

An asymmetric distribution of phospholipids exists between the two halves of many biological membranes of which the erythrocyte membrane is perhaps the best characterized (Op den Kamp, 1979; Rothman & Lenard, 1977). This raises the question of how asymmetry is maintained during the lifetime of the erythrocyte (120 days) when the half-time for transverse diffusion has been measured to be about 15 h (Van Meer & Op den Kamp, 1982). Although packing considerations would favour phospholipids with small head groups in the inner monolayer of sonicated vesicles which have tight curvature, this explanation would not hold for erythrocytes.

Early studies of transmembrane lipid redistribution (Kornberg & McConnell, 1971; McNamee & McConnell, 1973) showed that the rate of phospholipid transverse motion between the two halves of the bilayer is slow in model membranes but could be rapid in biological membranes. More recent studies (Seigneuret *et al.*, 1984) have shown that phospholipid asymmetry in erythrocyte membranes is ATP- and protein-dependent. A series of new spin labels were used of general structure:



where R = choline, ethanolamine or serine. These labels have the property of increased water solubility which allows ready labelling of the outer monolayer whilst having the nitroxide group near the surface and therefore accessible to the water-soluble reductants (e.g. ascorbate) used to monitor any redistribution of spin label after a chosen incubation period. The results showed that in erythrocytes, phosphatidylethanolamine and phosphatidylserine undergo rapid transverse diffusion from the outer monolayer leading to their preferential location in the inner monolayer; this process was reversibly inhibited following ATP depletion of the erythrocyte. By contrast, phosphatidylcholine remained in the outer monolayer and later studies showed that this was true also for sphingomyelin (Zachowski *et al.*, 1985). A protein, termed the aminophospholipid translocase, has been shown to be responsible and its lipid specificity has recently been examined (Morrot *et al.*, 1989). Another phospholipid translocase has been identified in endoplasmic reticulum vesicles from liver cells (Herrmann *et al.*, 1990); unlike the erythrocyte enzyme, the endoplasmic reticulum translocase exhibits only low specificity between different phospholipids. The properties of aminophospholipid translocases are quite different from those of phospholipid transfer proteins which catalyse lipid exchange between membranes; this exchange has also been studied by spin labelling e.s.r. (Wirtz *et al.*, 1980).

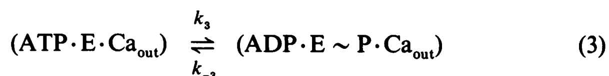
Spin label studies of membrane protein conformations and ligand-induced conformational changes

The number of studies of membrane protein conformation using the spin labelling technique is small compared with those analysing the conformation of the lipid. The aim is to label a specific amino acid residue (almost always cysteine) without inactivating the protein. If this aim can be realized, one may have a sensitive method for detecting ligand-induced conformational changes in membrane-bound enzymes, receptors and transport proteins. This approach is well illustrated by studies on the Ca^{2+}-ATPase in vesicles prepared from the sarcoplasmic reticulum membrane. Coan & Inesi (1977) showed that labelling of three cysteines in the ATPase lead to an e.s.r. spectrum with 'mobile' and 'less-mobile' components. It was found that labelling had little effect on ATPase activity or Ca^{2+} uptake. Addition of ATP in the presence of Ca^{2+} and Mg^{2+} left the 'mobile' component unchanged but converted part of the 'less-mobile' component to one that was more constrained; these spectral changes are reversed as ATP is hydrolysed. Derivatives of ATP where the terminal phosphate could not be hydrolysed gave similar spectral changes. The results were consistent with a conformational change in forming the Ca^{2+}–Mg-ATP–enzyme complex which precedes ATP hydrolysis and Ca^{2+} translocation, but the nature of the conformational states could not be fully defined. In later papers (Coan *et al.*, 1979; Coan & Keating, 1982; Coan, 1983), the labelling kinetics were studied by e.s.r. (cf. also Champeil *et al.*, 1978) in order to define conditions for more specific labelling, hence allowing a better characterization of the role of the 'constrained' conformation in the Ca^{2+} transport mechanism. The binding constant for Ca^{2+} determined by monitoring conversion to the constrained form in the presence of ATP agrees well with that for binding of Ca^{2+} to low-affinity sites on the vesicle interior. With derivatives of ATP that could not be hydrolysed, the constrained conformation is seen at low (micromolar) Ca^{2+} concentration, i.e. corresponding to high affinity sites on the vesicle exterior. This indicates that phosphorylation of the enzyme must be responsible for the changes in the Ca^{2+} affinity. Evidence based on the spin label data was also presented for two co-operative Ca^{2+} binding sites. These results can be discussed with respect to a proposed catalytic scheme (Fig. 4). The spectral changes observed with non-hydrolysable ATP derivatives correspond to steps 1 and 2, whilst the changes in the presence of Ca^{2+} and ATP correspond to the ADP·E-P forms of the enzyme shown in steps 4 and 5. The use of photoactivated 'caged' ATP (McCray & Trentham, 1989) in conjunction with spin-labelling of the Ca^{2+}-ATPase and other membrane proteins has great potential for kinetic and further mechanistic studies.

The alternative to spin-labelling the protein is to label a ligand, and studies on 3-hydroxybutyrate dehydrogenase using NAD(H) spin-labelled in the adenine ring offer a good illustration (Fritzsche *et al.*, 1984; Rudy *et al.*, 1989). 3-Hydroxybutyrate dehydrogenase is an enzyme that specifically requires phosphatidylcholine for activity. The spin-labelled nucleotide is a functional coenzyme and was found to be highly constrained when bound to the enzyme–phospholipid complex in the presence of substrate or inhibitor. Binding studies using e.s.r. on inhibitor–enzyme/phospholipid complexes revealed a stoichiometry of 0.5 per monomer (2 coenzyme molecules per tetramer).

$$ATP + ADP \cdot E_{out} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ATP \cdot E_{out} + ADP \quad (1)$$

$$ATP \cdot E_{out} + Ca^{2+}_{out} \overset{K_{Ca_{out}}}{\rightleftharpoons} (ATP \cdot E \cdot Ca_{out}) \quad (2)$$

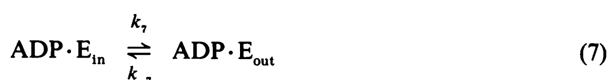


$$(ATP \cdot E \cdot Ca_{out}) \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} (ADP \cdot E \sim P \cdot Ca_{out}) \quad (3)$$

$$(ADP \cdot E \sim P \cdot Ca_{out}) \underset{k_{-4}}{\overset{k_4}{\rightleftharpoons}} (ADP \cdot E\text{-}P \cdot Ca_{in}) \quad (4)$$

$$ADP \cdot E\text{-}P \cdot Ca_{in} \overset{K_{Ca_{in}}}{\rightleftharpoons} ADP \cdot E_{in}\text{-}P + Ca^{2+}_{in} \quad (5)$$

$$ADP \cdot E_{in}\text{-}P + H_2O \underset{k_{-6}}{\overset{k_6}{\rightleftharpoons}} ADP \cdot E_{in} + P_i \quad (6)$$



$$ADP \cdot E_{in} \underset{k_{-7}}{\overset{k_7}{\rightleftharpoons}} ADP \cdot E_{out} \quad (7)$$

Fig. 4. Mechanistic scheme for active Ca^{2+} transport from outside to inside of the sarcoplasmic reticulum

E_{out} and E_{in} are the conformations of the Ca^{2+}-ATPase enzyme with the Ca^{2+} binding sites exposed to the outer and inner membrane surface, with high and low affinity, respectively. E ~ P and E-P are the high and low energy forms, respectively, of the phosphorylated enzyme intermediate. (Coan *et al.*, 1979).

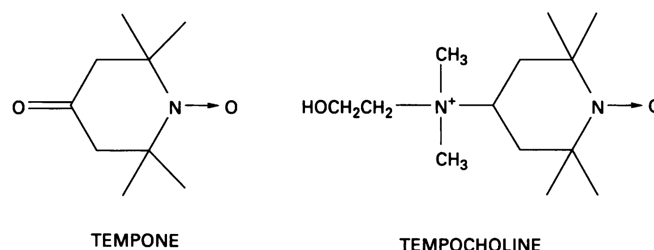
For complexes with weakly activating lipids, both the strength of binding and the degree of motional constraint of the spin-labelled nucleotide were reduced. The latter result suggests that phosphatidylcholine may act as an allosteric effector of 3-hydroxybutyrate dehydrogenase.

As a further example of ligand labelling, the studies of Oliveira *et al.* (1988) on interactions between sarcoplasmic reticulum ATPase and spin-labelled ATP derivatives may be cited. The results offer strong support for the proposal that catalytic and regulatory functions of ATP are effected through a single site.

A new era in studies of membrane protein conformation by e.s.r. seems probable following the report that cysteines could be introduced into defined positions in bacteriorhodopsin by using site-directed mutagenesis (Altenbach *et al.*, 1989, 1990). Mutant protein forms with single cysteines at 18 consecutive positions (residues 125–142) have been prepared and each has been specifically labelled with a spin probe. Studies of the e.s.r. relaxation behaviour in the presence of oxygen and water-soluble paramagnetic reagents indicate that residues 129–131 form a short water-exposed loop whilst residues 132–142 form a membrane-embedded α-helix. The orientation of the helix is established by the accessibility to oxygen concentrated in the lipid interior of the membrane, as illustrated in Fig. 5.

Spin label measurement of the internal volumes of vesicles and cells; studies of permeability

Measuring the uptake to equilibrium of spin labels such as TEMPONE or TEMPOCHOLINE is a useful method for determining the internal volumes of single-bilayer vesicles (Watts *et al.*, 1978) or cells (Melhorn *et al.*, 1982; Schobert & Marsh, 1982). The internal and external compartments are distinguished either by membrane-impermeant paramagnetic broadening reagents or by reduction of the external spin label with ascorbate.

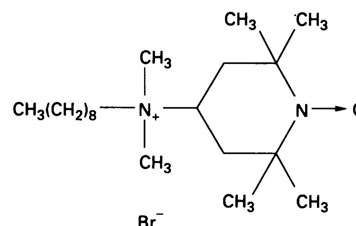


The time dependence of spin label uptake has been used to study permeability in lipid vesicles (Marsh *et al.*, 1976) and this was shown to be maximal at the gel–fluid phase transition. The permeability profile with temperature could be explained quantitatively by a mis-match in packing of ordered and fluid lipid regions. The wider biological implication is that similar mis-matching could occur at membrane interfaces (lipid–lipid, lipid–protein, etc.) in cells and thus be important in permeability and other membrane processes.

In this context of permeability, it is clear that n.m.r. is a powerful, non-invasive and selective method for studying ion channels *in vivo*. Ogino *et al.* (1983) showed by n.m.r. that K^+ efflux matched H^+ influx, but not Na^+ influx, on oxygenation of yeast cells. Yoon & Sharp (1985) established that Ca^{2+} influx is not linked to H^+ efflux in chromaffin granules. In leaf cells, n.m.r. can be used to distinguish chloroplast water from water in other cellular compartments and to measure the permeability coefficient for water from chloroplasts (McCain & Markley, 1985).

Measurement of surface potentials, membrane pH gradients and trans-membrane potentials

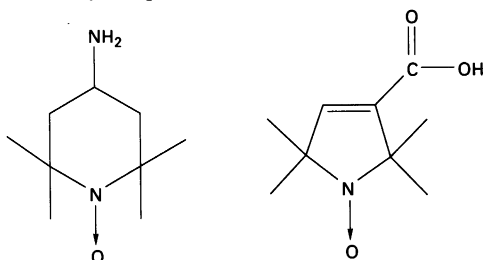
The theory and practice of these methods is covered in two recent reviews (Cafiso *et al.*, 1989; Cafiso, 1989). Measurement of surface potential, Φ, involves the use of charged paramagnetic amphiphiles such as (Castle & Hubbell, 1976):



which partition between the membrane and aqueous phases and give distinct e.s.r. spectra in these two environments. Negative charge at the membrane surface causes an enhancement in the surface concentration of the probe by the Boltzmann factor, exp $(-e\Phi/kT)$. Hence the electrostatic surface potential can be measured by e.s.r. spectroscopic determinations of the partition coefficient. In vesicles composed of charged and neutral phospholipids, the method gave reliable estimates of surface potential which were consistent with Gouy–Chapman theory (Castle & Hubbell, 1976), and the similar results obtained with oppositely charged probes further indicated the absence of any specific discrete-charge effects (Hartsel & Cafiso, 1986). Similar studies with phosphatidylcholine vesicles containing gangliosides, such as G_{M1} for which the charged oligosaccharide headgroup extends out into the aqueous phase, also gave reasonable values for the surface potential when allowance was made for the charge being located 1.0 nm beyond the surface (McDaniel *et al.*, 1986). For high surface charge densities there are significant deviations from Gouy–Chapman theory, as observed in a study of the ionic strength dependence of the phase transition temperature in phosphatidylglycerol bilayers (Cevc *et al.*, 1980). Studies on inner mitochondrial membrane vesicles

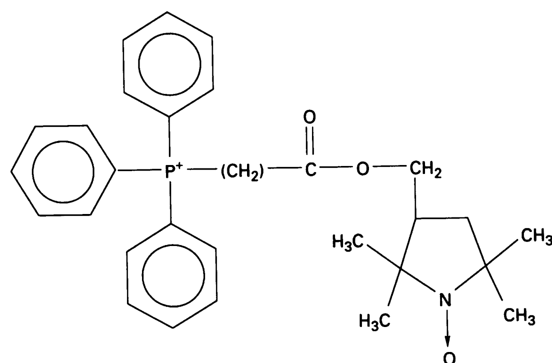
gave effects that were interpreted (Quintanilha & Packer, 1977) as changes in surface potential on energizing but which were insensitive to ionic strength. It is therefore likely that these effects may be attributable to changes in the membrane dipole potential (Cafiso & Hubbell, 1981; Flewelling & Hubbell, 1986*a*,*b*). An interesting extension of the method is to the study of asymmetry in surface potential across the membrane (Sundberg & Hubbell, 1986). In the case of measurements on the rod outer segment disc membrane (Tsui *et al.*, 1990) it was argued that the asymmetry in charged lipid distribution is induced by the bipolar distribution of charge on the transmembrane protein (Hubbell, 1990).

Transmembrane pH gradients (ΔpH) can be measured using amine or carboxylate probes such as:



which change their distribution across the membrane as a function of pH due to equilibration of the uncharged species coupled to the acid–base equilibrium. The external signal may then be removed using an impermeant paramagnetic broadening reagent such as ferricyanide. In thylakoids it was measured that a pH gradient of $\Delta pH = 3.2 \pm 0.2$ was generated on illumination (Quintanilha & Melhorn, 1978). Alternatively, paramagnetic amine amphiphiles have also been used for measuring pH gradients in lipid vesicles (Cafiso & Hubbell, 1978*a*).

Transmembrane potentials, ΔV, can be measured using spin-labelled hydrophobic ions such as (Cafiso & Hubbell, 1978*b*):



which distribute across the membrane according to the Nernst potential: $[SL]_{in}/[SL]_{out} = \exp(-e\Delta V/kT)$. This distribution is reflected in the water-membrane partitioning because of the higher surface to volume ratio within the vesicle. The method has been used to quantify transmembrane potentials in sonicated phosphatidylcholine mixed vesicles (Cafiso & Hubbell, 1978*b*) and to study the kinetics of the light-induced change in boundary potential (and transmembrane potential) in photoreceptor membranes (Cafiso & Hubbell, 1980). These hydrophobic ions have also been used to measure membrane currents to an accuracy of 1 pA/cm^2 (Cafiso & Hubbell, 1982, 1983) and to determine the membrane dipole potential from the much tighter binding of hydrophobic anions compared with cations in phosphatidylcholine vesicles (Flewelling & Hubbell, 1986*a*,*b*). It has also been demonstrated that the hydrophobic phosphonium ions bind to channel blocker sites in acetylcholine receptor membranes (Hartsel *et al.*, 1987).

In all probe studies of membranes, it is important to know the probe location in order to determine the region they are reporting. N.m.r. has been used to show that the above type of spin-labelled hydrophobic ion is located with its nitroxide adjacent to the first methylene group in the acyl chain (Ellena *et al.*, 1988).

PARAMAGNETIC CENTRES IN BIOLOGICAL MEMBRANES

Many biological membranes that support redox functions, notably the inner mitochondrial membrane and the thylakoid membrane of chloroplasts, have a diversity of paramagnetic species present. E.s.r. spectroscopy has been used to great effect since its advent in biology in the 1950's, not only in identifying these species but also in providing information on their location and orientation within the membrane (Beinert, 1978). It is also evident that now the structures of bacterial reaction centres are known at atomic resolution (Deisenhofer *et al.*, 1984; Allen *et al.*, 1986) there will be an increased interest in detailed e.s.r. studies designed to elucidate the mechanism of water splitting, charge separation and electron transfer processes involved in photosynthesis (Norris *et al.*, 1989).

E.s.r. used to identify paramagnetic species in membranes

The elegant use by Keilin of optical spectroscopy to identify and characterize cytochromes in intact respiratory chains is matched by the later use of e.s.r. to characterize iron–sulphur proteins in these membranes (Beinert, 1986). Unlike cytochromes, the optical absorbance spectra from iron–sulphur proteins are relatively featureless and their study necessitates the use of alternative spectroscopic methods, in particular e.s.r. The ability to poise the respiratory chain at a defined redox potential (Dutton & Wilson, 1974) meant that one could select which iron–sulphur species were present in paramagnetic form whilst in addition one could distinguish amongst these paramagnetic species according to their relaxation behaviour. A large number of iron–sulphur proteins have now been identified in respiratory chains (see, e.g., Ohnishi *et al.*, 1985*a*); indeed, the number found leads to questions as to their functional roles which certainly extends beyond participation as temporary electron sinks (Beinert, 1986).

The chlorophyll special pair involved in initiating photosynthetic electron transfer was first identified (Norris *et al.*, 1975; Feher *et al.*, 1975) through e.s.r. and ENDOR studies. These methods are still central to the study of intermediates in this pathway (Andreasson & Vanngard, 1988; Brudvig *et al.*, 1989).

E.s.r. has also been used extensively to study haem and copper centres in mitochondrial respiratory chains as typified by cytochrome oxidase (Vanngard, 1985; Li *et al.*, 1988), to study the manganese-containing protein involved in the water splitting reaction of photosynthesis (Brudvig *et al.*, 1989) and to characterize semi-quinone forms of redox cofactors, such as ubiquinone and flavin, in membranes (Edmondson & Tollin, 1983; Salerno *et al.*, 1990). It is now recognized that protein radicals have important and wide-ranging roles in biology (Stubbe, 1989); this class includes a tyrosine radical found in photosystem 2 of thylakoid membranes.

E.s.r. studies on the location and orientation of paramagnetic species in membranes

Knowledge of the location and relative orientation of redox centres in membrane-bound proteins is fundamental to a molecular understanding of the electron transfer processes. In the case of the bacterial photosynthetic reaction centre, this structural information is obtained through X-ray crystallography

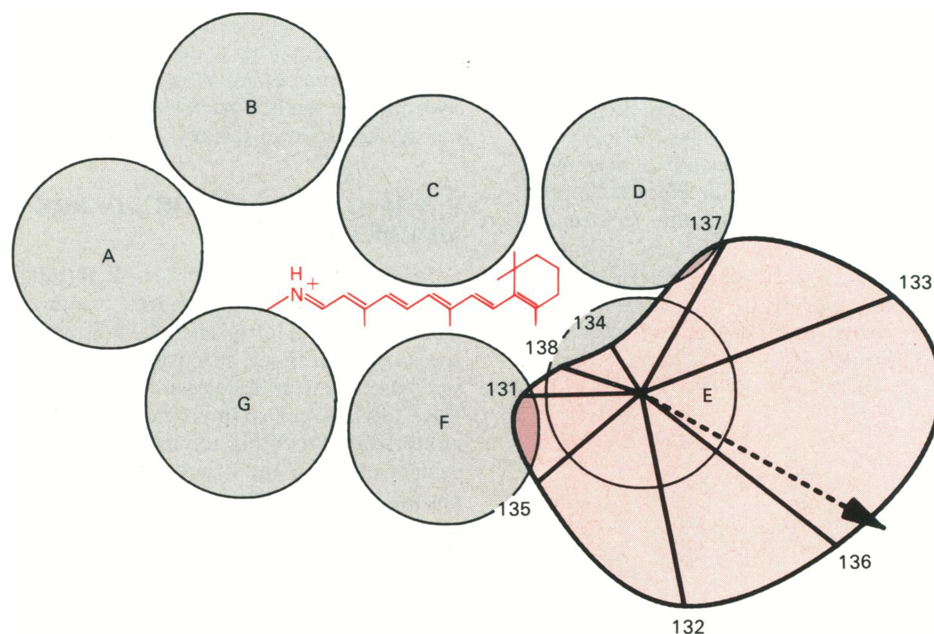


Fig. 5. α-Helical projection of the oxygen accessibility of residues 131–138 onto the low resolution structure of bacteriorhodopsin

The accessibility parameters for O_2 are drawn as vectors originating in the axis of helix E. The accessibility is greatest for amino acid residues facing the lipid bilayer and least for residues facing the protein interior. The results further establish a 3.6 residue periodicity in the accessibility parameter, corresponding to an α-helical secondary structure, and indicate a possible tilt in helix E. Reproduced with permission from Altenbach *et al.* (1990); © 1990 AAAS.

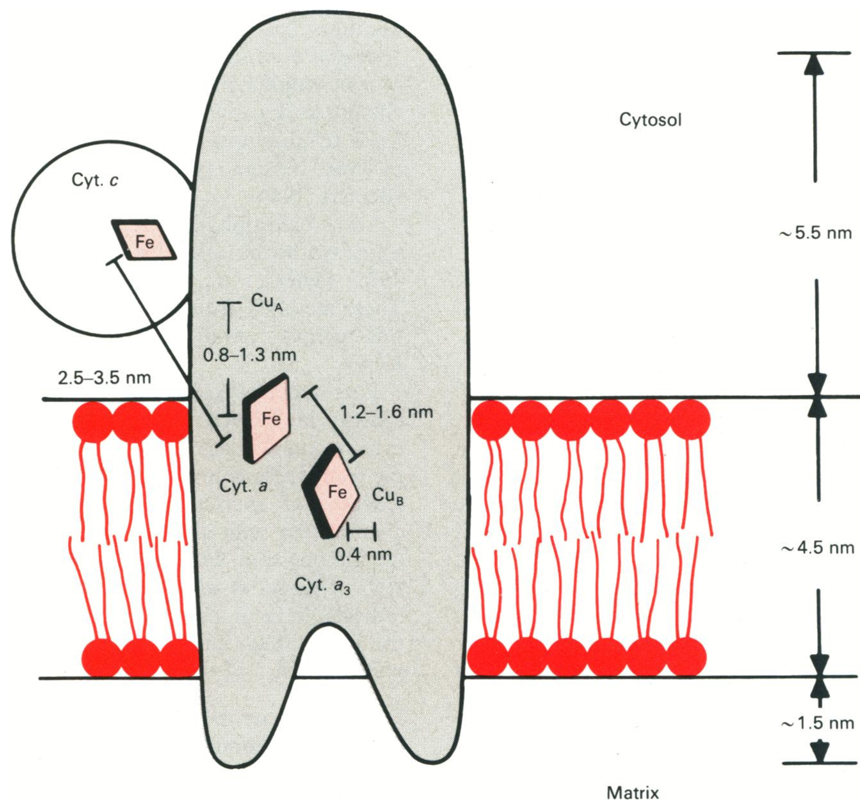


Fig. 6. Schematic diagram of the spatial organization of redox-active centres of cytochrome oxidase in the mitochondrial inner membrane

The separations of cytochrome *a* and cytochrome a_3 (Ohnishi *et al.*, 1982) and of Cu_A and cytochrome *a* (Goodman & Leigh, 1985) are deduced from e.s.r. measurements, as are the orientations of the haem groups (Erecinska *et al.*, 1977). (From Ohnishi *et al.*, 1985*b*).

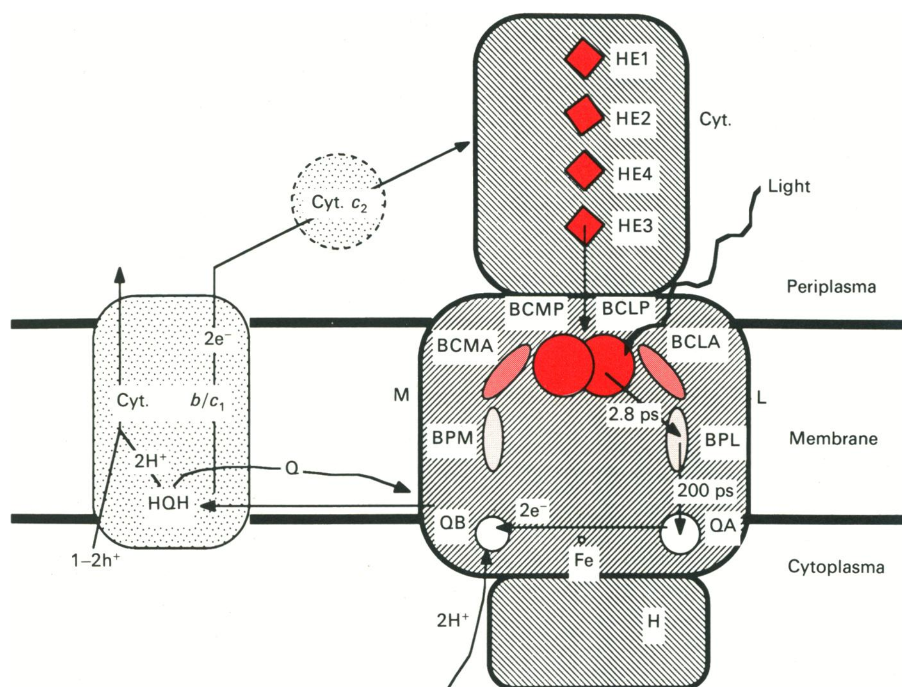


Fig. 7. The reaction centre of *Rhodopseudomonas viridis* showing the pathway of light-driven electron flow

The components of the bacteriochlorophyll dimer are indicated as BCMP and BCLP, where L and M are the two potential pathways for electron flow. BPL and BPM are the bacteriopheophytin molecules in the L and M arms, respectively, Q_A is menaquinone-9, and Fe indicates the non-haem iron. Reproduced with permission from Deisenhofer & Michael (1989); © 1989 The Nobel Foundation.

(Deisenhofer & Michel, 1989; Huber, 1989). An alternative approach for locating the redox sites utilizes dipolar interactions either between intrinsic paramagnetic sites or between an externally added paramagnetic probe and the paramagnetic site. For example, calculation of the distance between Cu_A and cytochrome *a* in cytochrome *c* oxidase is based on analysis of relaxation processes between these centres (Goodman & Leigh, 1985). The distance found of 0.8–1.3 nm (see Fig. 6) is internally consistent with other distance determinations including those of the distance from the membrane surface (Ohnishi *et al.*, 1985*b*). These latter determinations involve the use of Dy^{3+} complexes as membrane-insoluble relaxation probes to perturb paramagnetic sites in the protein. It has been found that the spin lattice relaxation rate of the selected paramagnetic site, as measured by the microwave power needed for half saturation of the signal, varies in inverse proportion with r^6 where r is the probe-to-paramagnetic-site distance. The studies by Prince (1983) on the Rieske iron–sulphur cluster in *Rhodopseudomonas sphaeroides* chromatophores are a good illustration of the method. Addition of Dy^{3+} relieves the microwave saturation behaviour of the cluster only when added from the cytochrome c_2 side of the membrane. The distance of the cluster from the cytochrome c_2 side of the membrane was estimated to be approx. 1.4 nm. It was further shown that the orientation of the iron–sulphur centre with respect to the membrane surface could be determined. The chromatophores can be oriented on a mylar plate and the variation in orientation of the principal axes of the *g*-tensor with respect to the magnetic field studied. The results establish that g_z, which corresponds to the Fe–Fe axis (Gibson *et al.*, 1966) of the cluster, lies in the membrane plane and the orientation is unaffected by addition of quinone inhibitors or reduction of the quinone pool.

Spectroscopic studies on crystals of the bacterial photosynthetic reaction centre give more detailed information on the electronic structures of the component species than is possible from studies of unoriented samples. Single-crystal e.s.r. studies have now been made on different paramagnetic species in the reaction centre, including the triplet state of the bacteriochlorophyll dimer primary donor (Norris *et al.*, 1989), the oxidized primary donor (Allen & Feher, 1984) and the reduced iron-quinone acceptor complex (Evelo *et al.*, 1988). One important question unanswered from the known three-dimensional structure of the reaction centre is why only one side, the L side (cf. Fig. 7), of the symmetrical complex is functional. The single-crystal studies by Norris *et al.* (1989) show that the operational symmetry of the centre is already broken in the primary donor, which establishes that the L-side nature of the photochemistry precedes even the reduction of bacteriopheophytin. Further e.s.r. studies may help in solving this intriguing puzzle.

CONCLUSIONS

The complexity of biological membranes

The drive behind magnetic resonance studies on membranes is to discover links between biophysical properties of these complex structures and function. In some cases, unique information can be obtained by direct studies on biological membranes despite their complexity. Thus the specificity of e.s.r. is well suited to unravel the complexities of intrinsic paramagnetic centres in mitochondrial membranes or the local behaviour of defined spin labels introduced into membranes, whilst n.m.r. studies of ^{13}C-labelled retinal incorporated into bacteriorhodopsin in purple membranes have the similar advantage of specificity. Many magnetic resonance studies on membranes have, however, been made on aqueous dispersions of polar lipids or on purified membrane proteins incorporated into model bilayer membranes. These limitations are largely dictated by the compositional

complexity of biological membranes and the need to simplify in order to have control over the experimental system. It is unlikely that such simple reconstituted systems support all the coupled activities of a particular protein, for example where the protein is a component of a multienzyme complex such as the mitochondrial respiratory chain; few magnetic resonance studies involving reconstitution of two proteins from a multienzyme complex have yet been attempted (e.g. Froud & Ragan, 1984). Again, the importance of coupling between factors is implied by the observation that reconstitution of transport systems often requires much higher lipid/protein ratios than are present in the biological membrane to achieve the permeability barrier with a single lipid class.

The functional requirements behind the structural complexity of biological membranes are not fully established but probably relate to maintaining appropriate local domains for the different protein components. This conclusion is supported by the relatively strict structural requirements for detergents and other species during the crystallization of membrane proteins (Roth *et al.*, 1989), although it must also be said that certain functional properties of individual reconstituted membrane proteins are tolerant of a diverse range of detergents and polar lipids. At present, information on domain or supramolecular structures in natural membranes and the role of specific associations (protein–protein, protein–lipid) in membrane function is lacking. Magnetic resonance methods are ideally suited to provide this type of information.

Magnetic resonance studies on membranes; achievements and difficulties

The achievements of magnetic resonance methods in providing a firm base for understanding the structural and dynamic features of membranes are impressive. The principles established have been shown to apply to both model and natural membranes. For example, lipid chain order parameter profiles (Seelig & Seelig, 1980; Stockton *et al.*, 1977), the properties of boundary lipid (Marsh & Watts, 1988; Bloom & Smith, 1985; Seelig *et al.*, 1982), quantitative information on protein rotational diffusion (Squier *et al.*, 1988*a-c*; Fajer *et al.*, 1989) and lipid lateral diffusion (Träuble & Sackmann, 1972; Devaux *et al.*, 1973; Rilfors *et al.*, 1986) have all been established through use of magnetic resonance methods. The difficulties faced by the magnetic resonance spectroscopist in answering questions on the functional consequences of these findings can be illustrated by two examples. Firstly, there have been many attractive proposals for the involvement of non-lamellar lipid structures in cellular processes such as fusion and permeability (Cullis & De Kruijff, 1979). Even though it is clear from magnetic resonance studies with model systems that certain naturally occurring polar lipids have a preference for a particular non-lamellar phase, biological membranes are of necessity lamellar. The reason obviously is to maintain the overall permeability barrier essential for membrane function, but the low frequency of occurrence and perhaps transient existence of possible non-lamellar lipid structures *in vivo* has hindered their detection by magnetic resonance methods (Seddon, 1990). The second example concerns the functional significance of specificity in protein–lipid interactions which has been detected by spin-labelling e.s.r. in such biological membranes as the rectal gland of *Squalus acanthias* (Esmann *et al.*, 1985) and thylakoid membranes of chloroplasts (Li *et al.*, 1989). Protein–lipid interactions detected by e.s.r. are well established biophysically (Marsh, 1983) and show clear specificity patterns with respect to polar lipid class. For the Na^+,K^+-ATPase from the rectal gland of *Squalus acanthias*, more efficient reconstitution of activity with certain acidic lipids is reported (Cornelius & Skou, 1984), which correlates broadly with the results on head group specificity from spin labelling studies (Esmann *et al.*, 1985) although other studies (Hilden & Hokin, 1976) report an active Na^+,K^+-ATPase preparation with phosphatidylcholine alone as the reconstituting lipid. The results indicate the difficulties in distinguishing a requirement for a particular lipid in supporting the activity of a particular protein and the less specific role of different lipids in the technology of reconstitution. With this system, more functional studies are called for in order to clear up discrepancies whereas in other cases where the functional requirement for a particular lipid class is strong, e.g. of phosphatidylcholine for 3-hydroxybutyrate dehydrogenase (Rudy *et al.*, 1989), more spin-labelling studies are needed. The links between specificity and function in protein-lipid interactions are discussed further by Marsh (1987).

Future prospects

The prospects for the future of magnetic resonance methods in the study of membranes are very promising. As the structures of more membrane proteins are determined by X-ray crystallography (Deisenhofer & Michel, 1989), electron diffraction (Henderson *et al.*, 1990), high resolution n.m.r. (Lee *et al.*, 1987; Tappin *et al.*, 1988; Schiksnis *et al.*, 1987; Smith & Griffin, 1988) and spin labelling e.s.r. in conjunction with site-directed mutagenesis (Altenbach *et al.*, 1990), the real power of spectroscopic methods in relating details of molecular structure and dynamics to function will become apparent. These studies will call on the full range of modern methods already introduced, including selective isotopic labelling of sites in both proteins and lipids and of advanced magnetic resonance methods, particularly solid-state n.m.r. and time-resolved e.s.r.

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