

Paramagnetic probes in membrane biophysics

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Abstract. The use of paramagnetic probes in membrane research is reviewed. Electron paramagnetic resonance studies on model and biological membranes doped with covalent and non-covalent spin-labels have been discussed with special emphasis on the methodology and the type of information obtainable on several important phenomena like membrane fluidity, lipid flip-flop, lateral diffusion of lipids, lipid phase separation, lipid bilayer phase transitions, lipid-protein interactions and membrane permeability. Nuclear magnetic resonance spectroscopy has also been effectively used to study the conformations of cation mediators across membranes and to analyse in detail the transmembrane ionic motions at the mechanistic level.

Keywords. Membranes; electron paramagnetic resonance; nuclear magnetic resonance; spin-labels; paramagnetic probes.

1. Introduction

Organization of living matter in cells is crucial for the sustenance of living beings. Membranes are primarily responsible for this organization giving rise to distinct organelles known as cells, mitochondria, ribosomes, nuclei etc. In addition to this compartmentalization, several cellular functions such as endocytosis, exocytosis, cell-cell communication, nerve-cell excitation, fertilization etc involve membranes in one way or the other. The chemical basis for the function of membranes lies in the amphipathic nature of lipids, which form the backbone of membrane structure. Chemically, biological membranes are made up of mostly proteins in addition to lipids. However, the nature of these proteins, called membrane proteins, is different from that of most other functional proteins. Membrane proteins are known to adopt conformations which are largely helical and to have hydrophobic exteriors.

Historically, understanding the membrane structure has been mainly aided by studies on model systems (Fendler 1982) like the monolayers at the air-water interfaces and micelles formed by surfactants and recently the lipid vesicles or liposomes. Surfactant chemistry has revealed the tendency of the amphipathic surfactants, lysolecithins and lipids to form aggregates. The hydrocarbon chains of these amphipaths come together, away from water, so as to form oily microenvironments exhibiting the so-called hydrophobic effect (Tanford 1973). Whereas certain amphipaths like most of the surfactants and lysolecithins form either monolayers or micelles, the double-chain lipids form inhomogeneous aqueous suspensions. These structures are multilamellar in morphology with an onion-like arrangement of several bilayers which break up on the application of ultrasonic irradiation to form small, closed bilayers called unilamellar vesicles (Bangham 1968). The micelles, reverse micelles, unilamellar and multilamellar vesicles are schematically represented in figure 1.

Arrangement of proteins in a lipid bilayer matrix is the significant feature of the presently accepted model for membranes (Singer and Nicolson 1972). In this model, proteins are either incorporated into the lipid bilayer (integral proteins) or mostly lie on

2. Spin-labels

Most of the spin-labels are derived from the oxazolidine and the piperidine or pyrrolidine rings (Rozantsev 1970) with the nitroxide group flanked by quaternary carbon atoms. Chemical structures of some spin-labels are shown in figure 2. The EPR spectra of these spin-labels (Berliner 1976) exhibit a three-line nitrogen hyperfine structure. The hyperfine splitting is dependent on the orientation of the magnetic field with respect to the nitroxide axes. When the motion of the spin-labels is fast compared with the spectral anisotropies, a three-line spectrum with equal line heights would be obtained. This corresponds to an isotropic correlation of time of 10^{-11} sec. As the motion becomes slow, there will be a differential broadening of the spectrum, with the line position unchanged. For still slower motions, the line positions and shapes get distorted. At the extreme end of complete immobilization, the so-called powder pattern is observed. In the case of fast but anisotropic motion, the spectral anisotropy is only partially averaged. In this case, the degree of motional averaging is a measure of the angular amplitude of anisotropic motion. These angular amplitudes are described in terms of order parameters. An order parameter of zero corresponds to an isotropic situation and it approaches unity with decreasing rates of motion.

3. Membrane fluidity

The nitroxide moiety can be covalently attached to the lipid, proteins and steroids. Several spin-labelled lipids and fatty acids have been synthesized with the label attached to the head group and at various carbon atoms of the hydrocarbon (Marsh 1981a). EPR

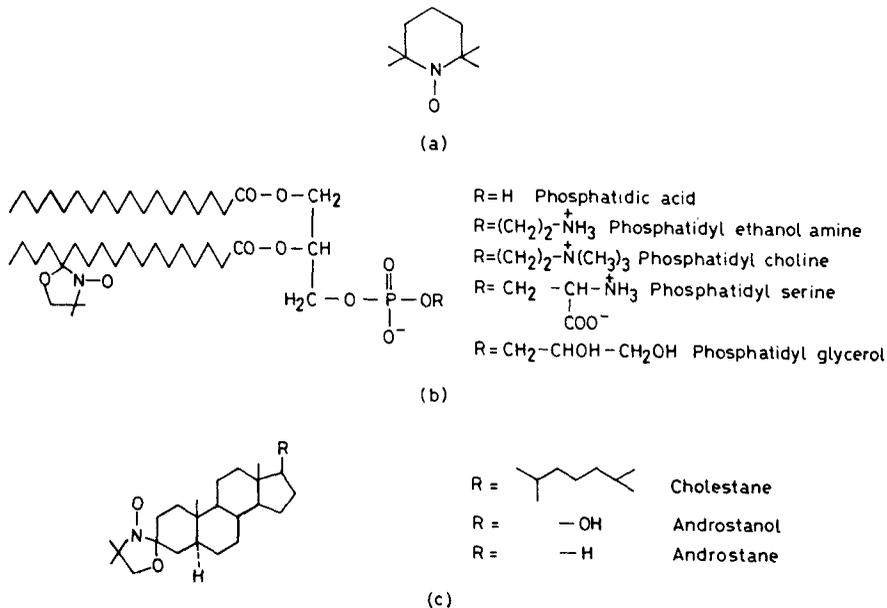


Figure 2. Chemical structures of some important spin-labels (a) 2,2,6,6, tetramethyl piperidin-1-oxyl (TEMPO), (b) β -14-(4,4-dimethyloxazolidinyl-N-oxy) stearyl- γ -acyl- α phospholipids and (c) spin-labelled steroids.

studies on egg phosphatidylcholine multilamellar dispersions doped with phosphatidylcholine spin-labels with the labels progressively going down the acyl chain have shown that the anisotropy of the spectrum, and hence the order parameter, decreases (Knowles *et al* 1976). This means that the motion increases as one goes from the head group to the interior, suggesting the existence of a flexibility gradient. The flexibility gradient arises because the lipid chains are anchored at the glycerol backbone of the lipid and the number of carbon-carbon single bonds that can undergo trans to gauche conversions increases down the chain (Marsh 1974). Several models have been proposed considering the interdependence of rotation about adjacent C-C single bonds (Seelig 1971), probabilities of trans to gauche conformers (Hubbell and McConnell 1971), intermolecular steric restrictions on the chain conformations (Marsh 1974) and interchain interactions (Marcelja 1974; Schindler and Seelig 1975) to account for the observed flexibility gradients and to calculate order parameters, probabilities of trans and gauche conformers, rotation potentials and several structural and thermodynamic properties.

Although EPR studies using spin-labelled lipids have provided a wealth of information, the perturbing effect of the nitroxide label group on the labelled chain and the surroundings has been considered a drawback. It has been argued that ^2H NMR provides a better source of information because ^2H is a non-perturbing probe (Schindler and Seelig 1975; Marsh 1981b).

4. Lateral diffusion

Lipid molecules freely diffuse laterally in the plane of the membrane. The rate of lateral diffusion is directly proportional to the collision frequency of two lipid molecules. When they are tagged by a spin-label, the inter-spin-label interactions would provide a measure of the lateral diffusion coefficients. For these experiments, concentrations of spin-labels used are normally higher than those used for other studies. At these concentrations, the spin-label-spin-label exchange interaction becomes appreciable leading to measurable interaction broadening. The dipolar and exchange contributions to the interaction broadening can be distinguished by line shape simulation and temperature dependence. Broadening could arise due to diffusion-controlled interactions or by label segregation. These two processes are easily distinguished by their different concentration dependence. Methods have been developed considering the process as a two-dimensional, homogeneous diffusion (Trauble and Sackmann 1972) or an exchange between nearest neighbours on a hexagonal lattice (Devaux *et al* 1973) to calculate diffusion coefficients from collision frequency. Lateral diffusion coefficients have been calculated for steroid spin-label in dipalmitoylphosphatidylcholine (DPPC) bilayers to be $10^{-8} \text{ cm}^2 \text{ sec}^{-1}$ (Trauble and Sackmann 1972) and for a phosphatidylcholine spin-label in sarcoplasmic reticulum membrane vesicles to be $6 \times 10^{-8} \text{ cm}^2 \text{ sec}^{-1}$ (Scandells *et al* 1972).

5. Lipid flip-flop

The transverse motion of lipids or a flip-flop of the lipid molecules between the two halves of the bilayer is much slower than the lateral diffusion rate accounting for the structural stability of a bilayer. This also leads to an asymmetric distribution of lipids as

observed in biological membranes. Lipid asymmetry can be measured by the addition of ascorbate at 0°C to a head group spin-labelled lipid containing vesicles. Ascorbate reduces the head group spin-labels on the outer half.

The intensities of the EPR lines before and after ascorbate reduction are proportional to the total lipid and lipid in the outer half respectively and hence can be used to calculate the fraction of lipids in the inner half. Values in the range of 0.25–0.35 are normally obtained for small sonicated vesicles and about 0.5 for large vesicles (Kornberg and McConnell 1971). Immediately after the ascorbate reduction, it can be eluted and the rate of flip-flop easily measured. The lipid flip-flop is considered a reversible, pseudo-unimolecular process. Experiments on egg phosphatidylcholine vesicles showed that the asymmetry decayed with a half-time of 6.5 hr, with probabilities of 0.07/hr and 0.04/hr for the outward and inward migrations. On the other hand, much faster rates (half-time 4–7 min) are observed for the excitable membrane vesicles from the electroplax of *Electrophorus electrans* (McNames and McConnell 1973).

6. Lipid phase separation

Certain lipid mixtures form bilayer structures but do not mix homogeneously. In this case, the measured exchange interaction can be used to analyse the lipid segregation in terms of the cluster size and density. Using a steroid spin-label, in DPPC bilayers below the phase transition temperature, a cluster density of $3.4 \times 10^{11} \text{ cm}^{-2}$ and an increase in the number of molecules per cluster from 25 to 150 with increasing concentration have been obtained (Trauble and Sackmann 1972). It was shown that PC and dipalmitoyl-phosphatidic acid (DPPA) phase separate in the presence of Ca^{2+} with a cluster density of $3.2 \times 10^{11} \text{ cm}^{-2}$ (Galla and Sackmann 1975).

Phase separation is easily detected using non-covalent labels which partition into a fluid membrane and practically do not enter a rigid interior. The free-radical 2,2,6,6-tetramethyl piperidin-1-oxyl (TEMPO) has been widely used to demonstrate solid phase and fluid phase immiscibilities (Shimshick and McConnell 1973).

7. Lipid bilayer phase transitions

Many aqueous dispersions of saturated lipids have a temperature below which the hydrocarbon chains are rigid. At a given temperature known as the phase transition temperature, the chains melt resulting in a cooperative phase transition from a gel to a liquid crystalline phase (Mabrey and Sturtevant 1978). This transition is accompanied by a sharp rise in the gauche conformers making the hydrocarbon interior fluid. Spin-labels like TEMPO are partitioned into the bilayer more when the bilayer is in the liquid crystalline phase than in the gel phase. EPR spectrum of TEMPO in hydrocarbons is different from that in water. This allows one to measure the uptake of TEMPO into the bilayer from the line heights and hence the phase transition temperature (Shimshick and McConnell 1973). The temperature dependent EPR spectra in DPPC multibilayers is shown in figure 3. Values obtained thereby are considered, on the one hand, to be in good agreement with those from differential scanning calorimetry but the differences also have been attributed to the perturbing effect of TEMPO and its specificity in interaction with the bilayer making it a less efficient probe.

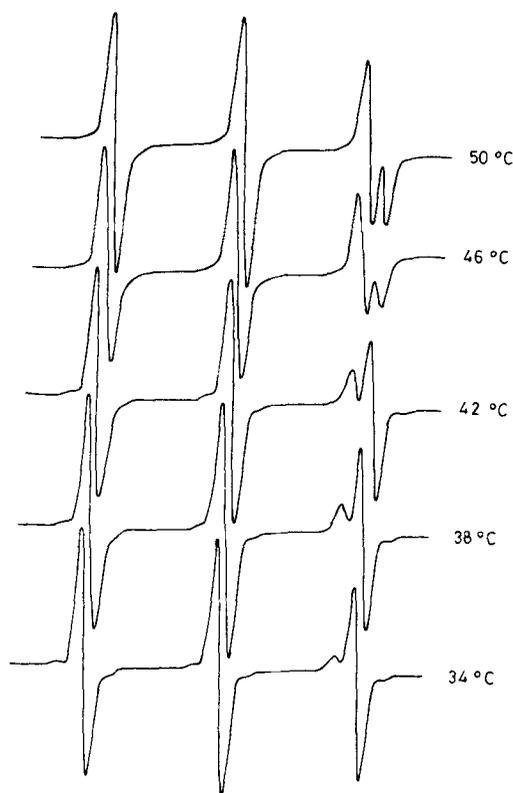


Figure 3. Electron paramagnetic spectra of TEMPO in DPPC multibilayers as a function of temperature (DPPC) = 0.18 M (TEMPO) = 0.75 mM phase.

Effects of additives on the phase transition have been studied using TEMPO partitioning EPR (Watts *et al* 1978). We have studied the lipid bilayer phase transition temperature of DPPC bilayers upon incorporation of the ionophore, valinomycin (Sankaram and Easwaran 1984). The phase transition temperature was unchanged upon addition of valinomycin but the pre-transition has been broadened (figure 4). These observations were attributed to a preferential location of the ionophore near the head group region. As the concentration of added valinomycin was increased, a decrease was observed in the uptake of TEMPO into the bilayer above the transition temperature ruling out any specific spin-label ionophore interactions. It appears that this is due to sharing of the available interior volume by both valinomycin and TEMPO (Sankaram 1983).

8. Lipid-protein interactions

Effects of membrane proteins on the organization of lipids in membranes have been successfully studied using spin-labelled lipids. Information on the stoichiometries of the lipid-protein complexes, specificity of lipid-protein interactions and the orders of selectivity has been obtained on several membrane proteins like cytochrome oxidase, rhodopsin, Na⁺/K⁺-ATPase, myelin proteolipid etc (Marsh 1983; Marsh *et al* 1982).

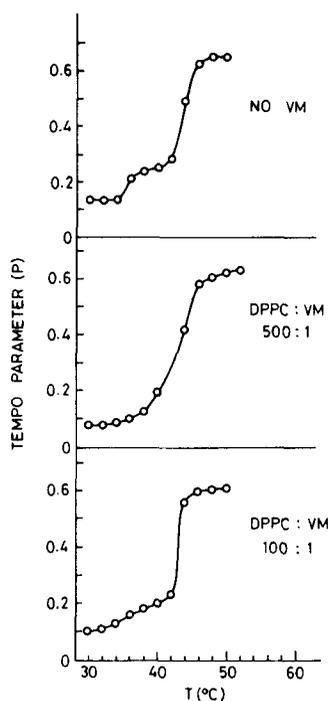


Figure 4. Transition temperatures of DPPC-VM multilayers by spin-label partitioning EPR (DPPC) = 0.18 M (TEMPO) = 0.75 mM.

Lipids labelled both at the head group and the hydrocarbon chains have been employed. The modes of interaction between the integral and peripheral membrane proteins have been found to be characteristic of their structures. The overall picture of a protein in a lipid environment is that of a motionally restricted component of the lipids around the protein with the local ordering decreasing as one goes away from the protein.

Membrane proteins can also be spin-labelled at the sulphhydryl groups which can be alkylated by maleimide and iodoacetamide nitroxide derivatives. EPR spectra of spin-labels covalently bound to the proteins in chromaffin granule membranes show strongly immobilized and small mobile components superimposed (Marsh *et al* 1976). The covalent protein labels have also been used to detect ligand-induced conformational changes in membrane-bound enzymes, receptors and transport proteins like Ca^{2+} -transporting ATPase of sarcoplasmic reticulum (Marsh 1981b).

9. Membrane permeability

The ascorbate reduction used in the lipid flip-flop studies can also be used for studying membrane permeabilities. In this case, the spin-label is added to the aqueous media and its permeability is measured. Permeability of TEMPO choline across DMPC bilayers has been measured in this way (Marsh *et al* 1976b). Trapped volumes of vesicles, vesicle permeability coefficients, diffusion coefficients and membrane potentials can be obtained by EPR studies of membrane permeabilities. Although EPR methods can, in

principle, be used for the study of membrane permeability, several important transmembrane transport processes cannot be conveniently studied. One such process namely the mediated cation transport can be conveniently studied using hydrophilic, paramagnetic probes in NMR.

Transmembrane cation transport is mediated by ionophores, membrane proteins and possibly by acidic phospholipids. The mechanisms of their action are classified as the diffusive carrier, relay carrier, channel and pore mechanisms which are illustrated in figure 5. The actual transport mechanism of a given system would be an appropriate admixture of these idealized cases. The diffusive carriers complex with the cation at one of the membrane-water interfaces and carry the cation to the other side by diffusion. The relay carrier mechanism involves, in addition to a diffusion of the carrier-cation complex, a handing over of the cation from one ionophore to the other. The carrier type ionophores valinomycin, nonactin, lasalocid, A23187 etc. act by these mechanisms (Ovchinnikov *et al* 1974). These ionophores complex with the cations to form equimolar and ion-sandwich complexes (Easwaran 1985; Vishwanath and Easwaran 1981, 1982, 1984; Shastri and Easwaran 1984). On the other hand, the channel and pore forming molecules bind to cations less strongly. They provide a hydrophilic interior which cations can pass through. Channels open randomly and per opening permit a given number of cations inside. A few such openings will saturate the inner pools. Pores allow a maximum number of cations sufficient to saturate the inner pools per opening.

Recent developments in the use of hydrophilic probes like lanthanides have permitted the use of NMR to study transmembrane ionic motions. Lanthanides when added to one side of the vesicle either shift the signals from the head group (phosphate and choline) on that side either upfield or downfield depending on the nature of the lanthanide or broaden them (Bystrov *et al* 1971). The effect of lanthanides on the ^1H NMR spectra of vesicles is shown in figure 6. Addition of a cation mediator to the vesicle sample containing the lanthanide on one side leads to a time-dependent increase in the intravesicular lanthanide ion concentration. Correspondingly, the NMR signals from the inside change their chemical shift, linewidth and intensity with time to approach the signals from the outside. The processes taking place during transport are shown in figure 7. The information on the mechanism of transport is contained in these changes (Ting *et al* 1981).

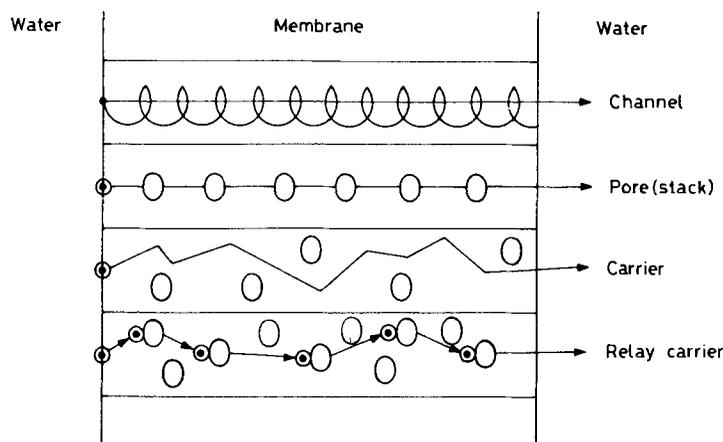


Figure 5. Mechanism of cation transport.

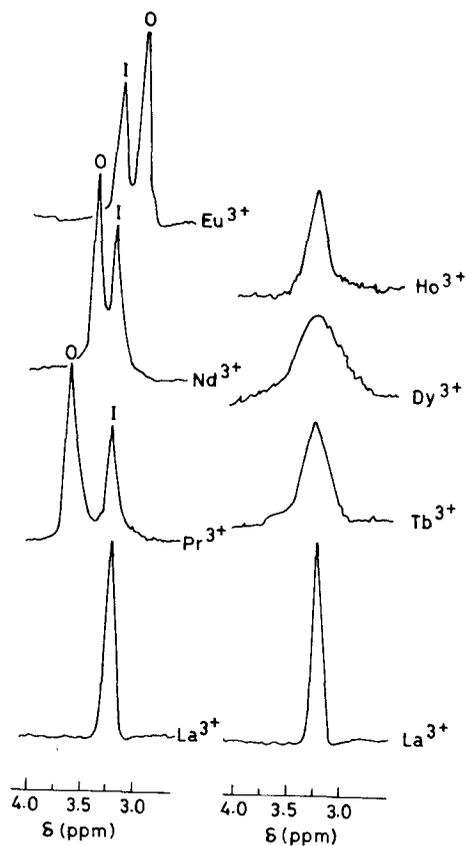


Figure 6. Effect of lanthanide addition on the ^1H NMR spectra of DMPC vesicles at 30°C .

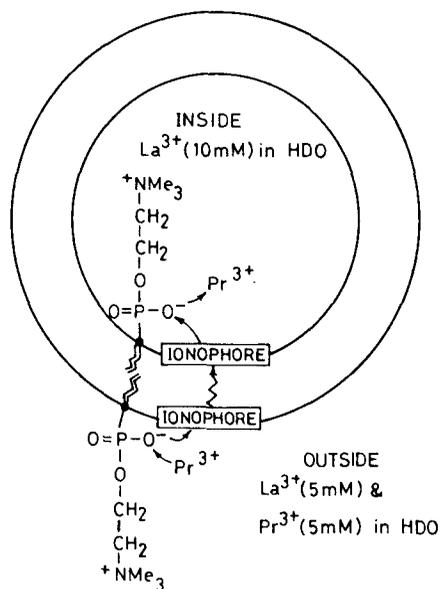


Figure 7. Strategy of the NMR kinetics experiments.

Using these methods, it has been shown that the carboxylic acid ionophores; lasalocid, A23187, etheromycin, tetronomycin, M139603, X-14547A and narasin (see Sankaram and Easwaran 1985a, b; Grandjean and Laszlo 1983) act by a diffusive carrier mechanism. These ionophores have been shown to transport Pr^{3+} orders of magnitude faster than the neutral class of ionophores due to the inability of proton antiport by the latter class. When two carboxylic ionophores are added together, they transport Pr^{3+} synergistically *i.e.* by a positive cooperative effect. Proton NMR spectral changes for the A23187 induced Pr^{3+} transport are shown in figure 8.

Lysolecithins and certain surfactants like triton X-100, bile salts form tetrameric structures to transport Pr^{3+} by an essentially diffusive mechanism. These tetrameric aggregates have been proposed to be reverse micellar in structures. Departures from transporting activity leading to perturbations in the membrane structure have been noticed in certain cases.

Pumping of cations in bursts, either a sufficient number per one opening or less, is manifested by a growth of new peaks in the NMR spectra without any chemical shift changes in the outer and inner signals. Growth of peaks corresponding to several partially filled inner pools at the expense of the inner choline signals has been observed for channels and pores. This kind of behaviour is exhibited for light induced Eu^{3+} transport by rhodopsin (O'Brien *et al* 1977) thermally-induced Eu^{3+} and Nd^{3+} permeation (Ting *et al* 1981) sonication-induced Pr^{3+} and Eu^{3+} permeation (Ting *et al* 1981) and cardiolipin-induced Pr^{3+} translocation (Sankaram *et al* 1985a). We have identified a borderline situation where the kinetics are carrier-like but the transporting

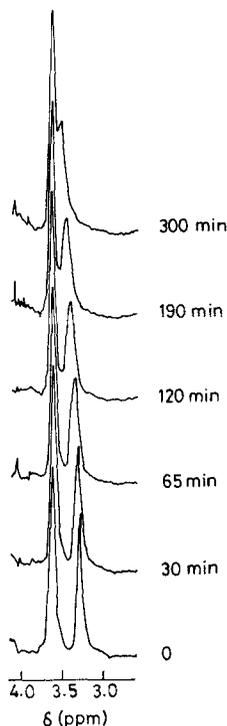


Figure 8. Time-dependent spectral change for A23187-induced Pr^{3+} translocation across DPPC vesicles at 60°C.

activity is a hexameric pore in the case of synthetic cyclo (L-Pro-gly)₃ mediated Pr³⁺ transport (Sankaram 1983).

10. Conformations of ionophores

Conformations of ionophores in solution are studied in detail by NMR spectroscopy. Paramagnetic probes have been used to delineate the intramolecular hydrogen bonding in peptide ionophores and to study the conformations of the cation complexes. Thus, the spin-label TEMPO has been used with success to detect the presence of intramolecular hydrogen bonds (Wuthrich 1976) in ion-binding cyclic peptides like valinomycin (Devarajan and Easwaran 1984). Presence of such a hydrogen bond leads to an absence of broadening of the amide protons upon addition of the spin-label and an appreciable broadening of the exposed amide protons. Use of TEMPO to delineate the hydrogen bonding in valinomycin and its K⁺ and Ba²⁺ complexes is shown in figure 9.

Pr³⁺ has been used to study the complexational characteristics of lasalocid by ¹H and ¹³C NMR to show that it forms an equimolar complex (Chen and Springer 1978). Our circular dichroism (CD) results on the lanthanide complexes of lasalocid show a dependence on the size of the cation—a manifestation of the lanthanide contraction (Shastri and Easwaran 1985). Gd³⁺ and Mn²⁺ have also been shown to bind to lasalocid (Hanna *et al* 1983) by their effect on the ¹³C NMR spin-lattice relaxation times. The binding sites have been shown to be determined by the cation charge and solvent polarity. We have also shown that valinomycin complexes with Mn²⁺ and forms both equimolar and ion-sandwich complexes by EPR spectroscopy (Sankaram and Easwaran 1982). The Mn²⁺ EPR spectral changes upon addition of valinomycin are shown in figure 10.

11. Conclusions

Paramagnetic probes *viz* covalently and non-covalently attached spin-labels, lanthanide ions etc are very useful probes of the structure and dynamics of membranes. Membrane fluidity, lipid motions, lipid bilayer phase transitions, membrane permeability and conformations of cation mediators can be studied in detail by the use of these probes in EPR and NMR spectroscopy. Further advances in the areas of

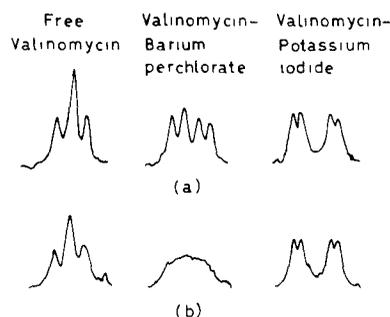


Figure 9. Effect of addition of TEMPO on the amide protons signals for (a) valinomycin (b) valinomycin-K⁺ complex and (c) valinomycin-Ba²⁺ complex in acetonitrile.

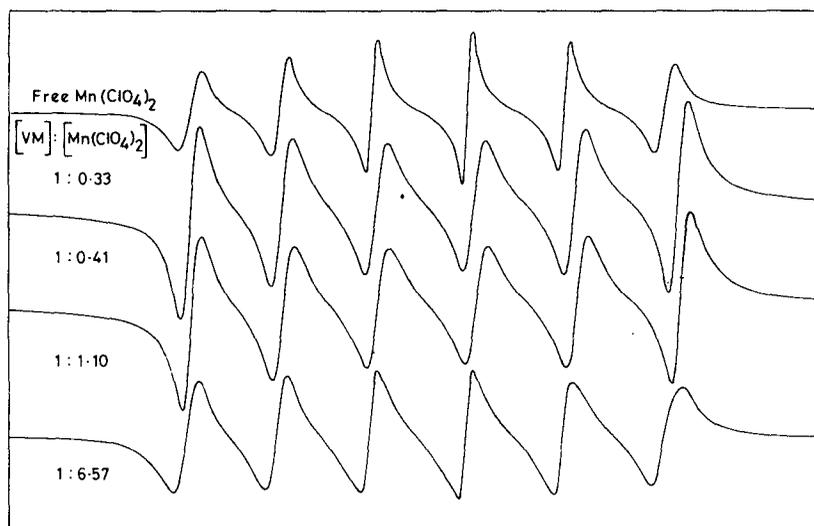


Figure 10. EPR spectra of Mn^{2+} upon addition of carrying amounts of valinomycin in acetonitrile at 25°C. $[Mn(ClO_4)_2] = 5$ mM.

lipid-protein interactions and transmembrane cation transport are rapidly taking place.

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