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ELECTRON SPIN RESONANCE OF BIOLOGICAL MEMBRANES: SPIN-LABELED LIPIDS AND PROTEINS

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I. INTRODUCTION

Electron spin resonance (ESR) and saturation transfer ESR (ST-ESR) have become established as biophysical techniques yielding valuable information on the structure and dynamics of lipids and proteins in biological systems. In this paper we review the 1985 to Spring 1987 literature centered around the topic "ESR of biological membranes". In reviews published during this period, much of the literature from before 1985 is treated extensively. These reviews are

listed in section 2 and for more information on specific topics the reader is referred to them.

Although computer-assisted literature searches have greatly alleviated the task of collecting relevant papers it is of course possible that we have missed one or more important papers in the field. We apologize for any omissions as well as for the fact that, unavoidably, emphasis in certain areas and lack thereof in others has been influenced by our own personal interests.

II. REVIEWS AND BOOKS

There are several recent reviews dealing with ESR applied to biological systems. A comprehensive series of reviews with emphasis on modern advances can be found in "EPR and advanced EPR studies of biological systems" [1], with literature through 1983. Chapters are dedicated to the theory of magnetic resonance, instrumentation and methodology of ESR, and applications of ESR spectroscopy to the study of protein structure and function, lipid membranes, and DNA. ESR-spin labeling and other methods employing physical labels are discussed by Likhtenstein et al. [2]. A brief overview of the use of spin labels in biochemistry is given by Watts [3]. Some of the theoretical concepts underlying spin label ESR spectroscopy and molecular mobility in biological systems are discussed by Marsh [4].

The structure and dynamics of biological membranes continues to be an area where application of electron spin magnetic resonance to a biological system appears most rewarding. This is clearly indicated by the many reviews specifically dedicated to this topic. Different aspects of spin label ESR spectroscopy pertaining to the molecular mobility of lipids and proteins in membranes and interactions between these membrane constituents are reviewed by Marsh, [5, 6], Davis [7], Devaux [8], Devaux and Seigneuret [9], Seigneuret et al. [10], Leaver and Chapman [11], Sankaram and Easwaran [12], and Swartz [13]. A review by Morse [14] not only provides a useful introduction to the ESR/spin labeling technique, but also discusses applications to biological membranes and new methodologies, for example, electron spin echo spectroscopy. Reviews by Smith and Butler [15], Fleischer and McIntyre [16], Hemminga [17], and Freed [18] contain sections in which special topics related to ESR of biological membranes are discussed. ST-ESR and its application to

the rotational diffusion of membrane proteins also are extensively reviewed by Thomas [19, 20, 21] and Thomas et al. [22].

III. FREELY-DIFFUSING SPIN LABELS

A. Lipid Spin Labels

The chemical structures of some examples of spin-labeled lipid analogues used in ESR studies of the structure and function of biological membranes are shown in Figures 1 and 2. Useful lipid spin probes include: spin-labeled fatty acids (Figure 1 a, b, c); spin-labeled, charged amphiphiles (Figure 1 d, e, f); spin-labeled steroids (Figure 1 g, h, i); and spin-labeled phospholipids (Figure 2).

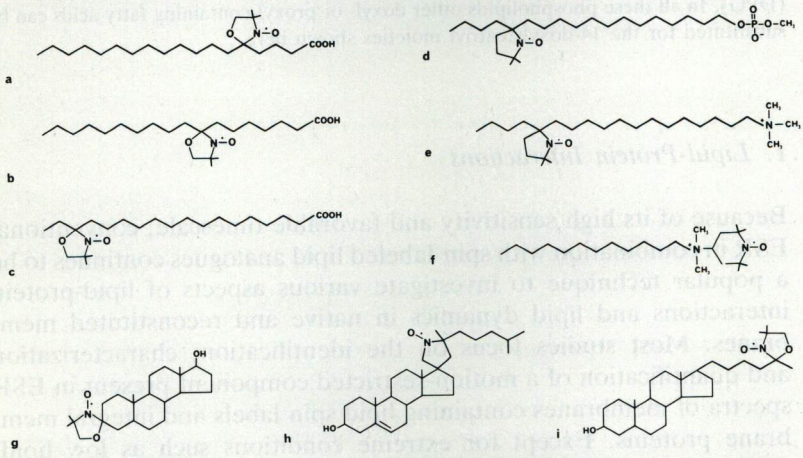

FIGURE 1 Chemical structures of single-tail and steroid lipid spin labels useful for investigation of the dynamic behavior of lipids in biological membranes by ESR. Structures a, b, and c are examples of spin-labeled fatty acids which are the most commonly used spin probes. By varying the position of the nitroxide moiety along the alkyl chain, segmental motion and order can be examined as a function of bilayer depth. The chemically more stable proxyl group (see d and e) can be used instead of the doxyl group as the nitroxide-bearing moiety. Structures d, e, and f are examples of spin-labeled, charged amphiphiles. Structures g, h, and i are examples of spin-labeled steroids which are useful for study of cholesterol and analogues. The names of the spin labels shown are: a, 5-doxylstearic acid; b, 8-doxylstearic acid; c, 16-doxylstearic acid; d, 14-proxylstearylmethyl phosphate; e, N-14-proxylstearyl-N,N,N-trimethylammonium; f, N,N-dimethyl-N-dodecyltempoylammonium; g, 3-doxyl-androstanol; h, 17, 2′-pyrollidine cholesterol; i, 25-doxylcholestanol.

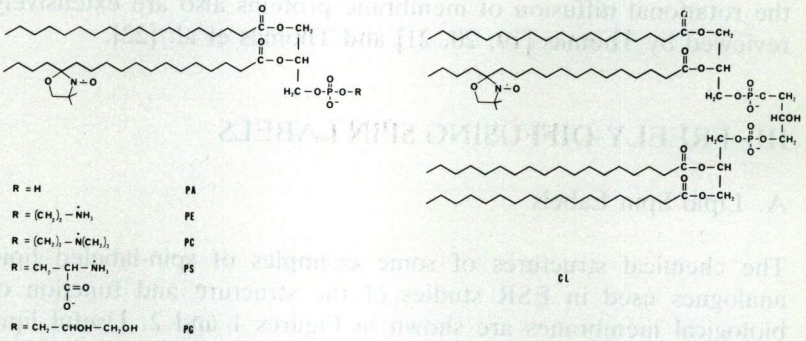

FIGURE 2 Chemical structures of spin-labeled phospholipid analogues: PA, phosphatidic acid; PE, phosphatidylethanolamine; PC, phosphatidylcholine; PS, phosphatidylserine; PG, phosphatidylglycerol; CL, cardiolipin or diphosphatidylglycerol (DPG). In all these phospholipids other doxyl- or proxyl-containing fatty acids can be substituted for the 14-doxylstearoyl moieties shown here.

1. Lipid-Protein Interactions

Because of its high sensitivity and favorable timescale, conventional ESR in combination with spin-labeled lipid analogues continues to be a popular technique to investigate various aspects of lipid-protein interactions and lipid dynamics in native and reconstituted membranes. Most studies focus on the identification, characterization and quantification of a motion-restricted component present in ESR spectra of membranes containing lipid spin labels and integral membrane proteins. Except for extreme conditions such as low lipid-protein ratios or temperatures below the hydrocarbon chain melting transition of the lipids, this motion-restricted component arises from spin-labeled lipids in contact with the hydrophobic protein surface. [We recommend use of the term "*motion-restricted component*" rather than "*immobilized component*". Although well known in spectroscopy, the latter term may give rise to the erroneous concept of a "permanent annulus of immobilized lipids" surrounding a membrane protein. Quite to the contrary, lipids in the boundary layer have been shown to retain considerable motion and to exchange with the bulk bilayer lipids with a rate that is slow to intermediate on the ESR timescale but fast, for instance, on the NMR timescale (see ref. 6).]

Seigneuret et al. [23] have studied cholesterol-protein interactions in the human erythrocyte membrane using a spin label analogue of cholesterol bearing a nitroxide on the alkyl chain (26-nor-25-doxylcholestanol). The ESR spectrum is composed of a mobile component and a highly motion-restricted component (outer hyperfine splitting 61−63 G) representing about 45% of the signal. Removal of the cytoskeletal proteins reduces the motion-restricted component, while it is no longer observed after chymotrypsin treatment which reduces band 3 protein to a smaller fragment. These results are consistent with a previously proposed selective band 3-cholesterol interaction and with the known linkage between band 3 and the erythrocyte cytoskeleton.

From the same laboratory there also appeared a series of studies on the transbilayer diffusion of phospholipids in human erythrocytes. Zachowski et al. [24] use spin-labeled analogues of phosphatidylcholine, phosphatidylethanolamine and phosphatidylserine to compare the transverse diffusion rates of lipids in normal and sickle erythrocytes. Rapid incorporation of these lipid spin labels into the cell membrane was facilitated by the presence of a short, nitroxide-bearing acyl chain in the *sn*-2 position, but such a structural modification may, of course, affect transbilayer diffusion as well. By selective chemical reduction of the nitroxide labels embedded in the outer leaflet of the membrane it was shown that the aminophospholipid analogues, but not the phosphatidylcholine analogue, diffuse toward the inner leaflet within 3 h. The transverse diffusion rate of the amino-analogue is significantly reduced in homozygote sickle cells when compared with normal cells. In a separate report Zachowski et al. [25] show that a spin-labeled analogue of phosphatidic acid diffuses slowly, similar to the phosphatidylcholine analogue, while a spin-labeled sphingomyelin analogue shows no inward transport. More recent papers from Devaux's group provide evidence that the outside-inside translocation of aminophospholipids in the human erythrocyte membrane [26], in the pig lymphocyte plasma membrane [27], and in human platelets [28] is a protein-mediated phenomenon. It remains to be shown, however, that translocation of natural aminophospholipids, which carry two long acyl chains, occurs in a manner similar to that observed for the short-chain spin-labeled analogues.

An interesting study is that of Fong and McNamee [29], who attempt to correlate *Torpedo californica* acetylcholine receptor function with structural and dynamic properties of the host lipid

bilayer. The low to high agonist affinity-state transition in the presence of carbamylcholine and the ion-gating activity of receptor-containing vesicles in response to carbamylcholine were measured as a function of the bilayer lipid composition. Dynamic properties of these membranes were determined by incorporating spin-labeled fatty acid and measuring the order parameter. It is found that an optimal membrane fluidity (i.e. lipid segmental motion) is required for interconversion between the high and low affinity states of the acetylcholine receptor. Ion-gating activity, however, requires the presence of both cholesterol and negatively charged phospholipids in addition to optimal fluidity. The acetylcholine receptor thus appears to be an example of a membrane protein for which structural and dynamic requirements of the host lipids are clearly implicated in biological function.

Tanaka and Freed [30] have studied lipid-gramicidin A interactions utilizing macroscopically aligned samples of low ($< 10\%$) water content and ESR of spin-labeled lipids and a cholestane probe. Freed and coworkers continue to express some reservations regarding the interpretation of the two-component ESR spectra obtained for protein-containing lipid bilayers in terms of a two-site model. However, from their results on the oriented lipid bilayers Tanaka and Freed conclude that the main lipid-gramicidin A interaction is that of a boundary effect. The polypeptide induces disorder at low temperature and low water content but induces order at high temperature and high water content, while lipid fluidity is only slightly affected.

Lipid mobility and order in bovine rod outer segment disk membranes have been studied by Pates and Marsh [31] using a series of eight stearic acid spin label probes labeled at different carbon atom positions in the chain. All spectra consist of two components; one closely resembling the spectra obtained from dispersions of the extracted membrane lipids and the other, characterized by a considerably greater degree of motional restriction, induced by the presence of rhodopsin. The proportion of the motionally restricted lipid component is approximately constant and independent of the position of the spin label group. The spectral characteristics of the motionally restricted component as a function of temperature indicate that the mobility of these lipids in contact with protein increases with temperature but does not vary greatly along the middle portion of the chain, between carbons 8 and 14 from the carboxyl

group. ESR spectra obtained as a function of the direction of the magnetic field with oriented samples indicate that the motionally restricted acyl chains have a broad distribution of orientations. It is concluded that the spin-labeled lipids in direct association with rhodoposin are not highly ordered and display motional restriction along much of the length of their chains. This result is similar to the early observations on lipid interactions with the mitochondrial protein, cytochrome *c* oxidase [32, 33, 6].

The effect on the lipid order parameter of microsomal cytochrome P-450 and NADPH-cytochrome P-450 reductase reconstituted in unilamellar phospholipid vesicles has been investigated by Kunz et al. [34], employing fluorescence and ESR. The fluorescent probe used was 1,6-diphenyl-1,3,5,-hexatriene and the spin probes were phosphatidylcholines with 5- or 8-doxylstearic acid esterified at the *sn*-2 position. Both techniques show that these proteins decrease the order parameter of the phospholipid acyl chains, contrary to what has been observed for other proteins, e.g., bacteriorhodopsin and cytochrome *c* oxidase.

Volotovski et al. [35] have determined the effect of calmodulin on the order parameter of lipids in photoreceptor membranes and rhodopsin-containing phospholipid vesicles by spin label ESR. Calmodulin did not change the order parameter of lipid spin labels in bilayers containing only rhodopsin but induced a significant increase in the order of the lipids in bovine rod outer segment disk membranes, which contain rhodopsin and other proteins. This suggests that the site of calmodulin binding is remote from rhodopsin itself.

Nishiyama and Kuninori [36] have looked at the effect of oxygen on wheat flour lipids during dough mixing. Dough was prepared in the presence of the spin label 5-doxylstearic acid under different nitrogen/oxygen atmospheres and ESR spectra of the gluten were recorded. The ESR data combined with lipid analysis indicate that modification of the lipids by oxidation leads to increased fluidity and a decrease in lipid-protein interactions. These observations may relate to the effect of oxygen and lipid oxidation on the rheological properties of dough.

In addition to studies of lipid-protein interactions involving integral membrane proteins, other groups have employed ESR to investigate lipid binding of water-soluble proteins and peptides. Rietveld et al. [37, 38] and Goerrissen et al. [39] have studied the

association of apocytochrome *c*, the non-heme precursor of the mitochondrial protein cytochrome *c*, with model membranes consisting of or containing negatively charged phospholipids. It is shown that upon electrostatic interaction with the negatively charged lipids, apocytochrome *c* is able to penetrate into the hydrophobic region of the membrane. ESR spectra of phospholipids spin-labeled at different positions along the *sn*-2 chain indicate a generalized decrease in mobility of the lipids but the characteristic flexibility gradient toward the terminal methyl end of the chain is maintained. With lipids spin-labeled close to the terminal methyl end a second, more motionally restricted component is observed with apocytochrome *c* but not with cytochrome *c*, indicating a fundamental difference in the mode of binding of these proteins to negatively charged lipids. In mixed model membranes consisting of negatively charged and neutral lipids, the protein interacts preferentially with the negatively charged lipids but this preferential interaction does not induce separation of large domains enriched in complexes of apocytochrome *c* and negatively charged phospholipids and domains enriched in neutral lipids.

The activation of the lipolytic enzyme, porcine pancreatic phospholipase A_2, by the presence of negative charges at the lipid substrate-water interface has been investigated by Volwerk et al. [40] using a combination of kinetic studies, fluorescence, and ESR experiments. The strong interaction of this enzyme with negatively charged lipids present in aqueous solution or in mixed micelles was confirmed using both negatively and positively charged spin-labeled detergent analogues. These results have physiological implications because the natural substrates of this digestive enzyme are mixed micelles containing long-chain phospholipids and negatively charged detergent-like bile salts. The data show clearly that charge interactions between the negatively charged micelle and positive amino acid residues of the enzyme facilitate efficient hydrolysis of the solubilized lipid substrate.

Surewicz and Epand [41] examine the effects of amino acid substitutions in the pentapeptide pentagastrin on the nature of its interactions with dimyristoylphosphatidylcholine (DMPC) liposomes by differential scanning calorimetry (DSC) and ESR. Pentagastrin is a biologically active, chemically modified form of the carboxyl-terminal tetrapeptide of the hormone gastrin. In two peptide analogues, the Asp at position 4 in pentagastrin was replaced by Gly

or Phe, resulting in uncharged and more hydrophobic peptides. Three charged analogues were also studied: pentagastrin itself, an analogue with positions 4 and 5 reversed, and one with Asp-4 replaced by Arg. These peptides mimic the behavior of integral membrane proteins both in their effect on the DMPC phase behavior as reported by DSC, and their effect on lipid mobility as reported by stearic acid spin probes. It is concluded that peptide composition, charge, and sequence are important in determining the nature of peptide-lipid interactions.

Ahmad et al. [42] have studied the association of apolipoprotein C-III (apoC-III) from human very low density lipoprotein with egg yolk sphingomyelin at the transition temperature of the phospholipid. The combined results from circular dichroism, gel filtration, sedimentation velocity experiments, quenching of the tryptophan fluorescence by spin-labeled phosphatidylcholine, differential scanning calorimetry and electron microscopy indicate that apoC-III binds to the sphingomyelin vesicles inducing a structural transition to much smaller particles. A model is presented featuring a disc-like cylindrical bilayer whose outermost rings of acyl and alkenyl chains are covered by apoC-III with the helical axis of this protein perpendicular to the long axis of the chains.

Mims et al. [43] use a series of spin-labeled phosphatidylcholines (PC) and cholesteryl esters (CE) with a nitroxide group at fatty acyl carbon C5, C12, or C16 to study acyl chain motions at the surface or within the core of microemulsion particles (ME) with or without apolipoprotein E (apoE) bound to their surfaces. These particles are about 750 Angstroms in diameter, contain cholesteryl oleate and are coated with a monolayer of dimyristoylphosphatidylcholine (DMPC). ESR data obtained with the spin-labeled PC's indicated a gradient of motion in the ME surface monolayer similar to that observed with the same probes in a bilayer. The 5- and 12-doxyl CE's demonstrated a higher degree of order for the CE-rich core than the corresponding PC's showed for the phospholipid-rich surface. Binding of apoE to spin-labeled DMPC vesicles increased the order of the 5-position of the *sn*-2 chain but for the ME particles the effect of protein binding was not as striking. Ascorbate reduction indicated that the C5-position of the *sn*-2 chain of PC in the microemulsion was less accessible than the corresponding position in the vesicle.

Nöthig-Laslo and Knipping [44] compare lipid dynamics at the

surface layer of porcine and human high density lipoprotein (HDL) subclasses by spin labeling. Porcine HDL was artificially subdivided into buoyant density subclasses corresponding to those of human HDL and the motion of four different positional isomers of spin-labeled fatty acids and of spin-labeled androstanol, introduced into these HDL subclasses, was determined by ESR. In general, the spin labels experienced more restricted motion in the porcine HDL than in the human HDL_2 and HDL_3 subclasses.

Baglioni et al. [45] have studied two-dimensional mixtures of a polypeptide, poly (γ-methyl-L-glutamate), and the spin probes, 5-doxylstearic acid and 16-doxylstearic acid, in monolayers at the air-water interface. Interactions between each spin label and the polypeptide were investigated by analyzing the parameters obtained from the spreading isotherms and by analyzing ESR spectra of the collapsed monolayers. The position of the nitroxide group along the hydrophobic chain affected the interaction of the probe with the polypeptide, indicated by differences in the Gibbs free energy of mixing.

2. *Multiple Binding Equilibria of Lipid-Protein Interactions*

Besides the mostly qualitative studies discussed above, more quantitative ESR-spin labeling studies aimed at determining thermodynamic parameters of the interactions between integral membrane proteins and membrane phospholipids continued to appear during the review period. Pates et al. [46] have employed freely diffusing phospholipid spin labels to study rhodopsin-lipid interactions in frog rod outer segment membranes. Each of the spin probes shows a two-component ESR spectrum with a motionally restricted component attributed to spin labels in contact with the surface of rhodopsin and the other component originating from spin labels in the fluid lipid bilayer region of the membranes. The population of motion restricted lipids is sufficient to coat the hydrophobic protein surface while there is little selectivity beween the different spin-labeled phospholipid classes examined.

From the same laboratory appeared a spin-label study of lipid-protein interactions in Na^+, K^+-ATPase membranes from *Squalus acanthias* [47, 48]. Using lipids with nitroxide labels at the 14-position of the *sn*-2 chain, Esmann and Marsh [47] have investigated the pH

and salt dependence of the interactions of phosphatidic acid, phosphatidylserine and stearic acid with the ATPase. For phosphatidic acid and stearic acid, the fraction of motionally restricted spin label increases with pH, with pK_a's of 6.6 and 8.0, respecively, whereas the fraction of motionally restricted phosphatidylserine spin label remains constant between pH 4.7–9.2. Similarly, high salt decreases the fraction motionally restricted component for phosphatidic and stearic acid but has relatively little effect on that of phosphatidylserine. The authors conclude that direct electrostatic effects alone cannot account for all of these observations. In a second paper Esmann et al. [48] show that the fraction of motion restricted lipids corresponds to a stoichiometry of aproximately 66 lipids per 265,000-dalton protein. This number of lipid molecules roughly can be accommodated within the first shell around the protein dimer. The ATPase shows some selectivity for association with the various spin-labeled lipids in the order: cardiolipin > other negatively charged phospholipids > phosphatidylcholine ≅ androstanol.

The association of spin-labeled cardiolipin with bovine heart cytochrome *c* oxidase reconstituted in dimyristoylphosphatidylcholine has been investigated by Powell et al. [49]. They report a lipid-substituted preparation which contains less than one mole of cardiolipin per mole of enzyme but, nevertheless, retains oxidative activity. ESR spectroscopy of these lipid-substituted cytochrome oxidase preparations demonstrates an average association constant for cardiolipin that is 5.4-times that for phosphatidylcholine. This selectivity is interpreted as corresponding to a generalized increase in specificity for all lipid association sites on the protein.

Some advances toward a better understanding of the specificity of lipid-protein interactions have been made recently [50]. In this study the model for lipid-protein interactions previously described by Brotherus et al. [51] is generalized to include lipid binding to an integral membrane protein solubilized in excess detergent. The central assumption in this multiple equilibria binding treatment is that in lipid bilayers and in lipid/detergent mixed micelles there is a competition between lipid and detergent molecules, for the hydrophobic protein surface, as diagrammed in Figure 3. Competition between lipid (L) and detergent (D) molecules in a protein-containing mixed micelle is represented as a series of exchange reactions at the hydrophobic protein-lipid interface:

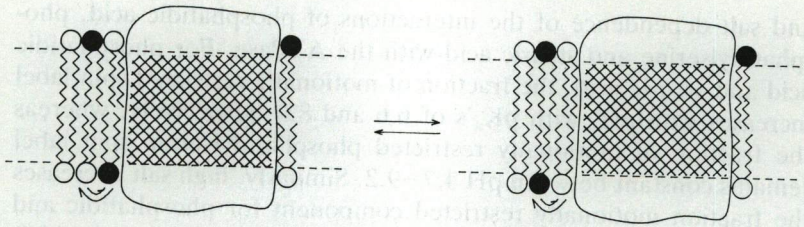

FIGURE 3 Schematic drawing of the dynamic exchange of phospholipid molecules at the protein-lipid interface of a membrane protein in a lipid bilayer. A similar exchange occurs between lipid and detergent molecules when a membrane protein is present in a lipid/detergent mixed micelle. The crosshatched area indicates the hydrophobic protein surface normally exposed to the lipid hydrocarbon chains. For phospholipid molecules situated adjacent to a protein molecule, any restriction of acyl chain motion imposed by the protein surface is reported by the nitroxide spin label moiety attached near the terminal end of one of the acyl chains. This interaction between lipid and protein gives rise to the motion-restricted component observed in the ESR spectrum.

$$BD_\beta + L \rightleftharpoons BL + \beta\ D$$

where B is a contact site and β represents the number of detergent molecules replaced by a single lipid molecule. For a protein with multiple classes of contact sites the following general binding equation is derived:

$$\bar{\nu} = \sum_{i=1}^{m} \frac{N_i K_i X_{Lf} X_{Df}^{-\beta}}{1 + K_i X_{Lf} X_{Df}^{-\beta}}$$

where $\bar{\nu}$ is moles of lipid bound per mole of protein, N_i and K_i are the number of contact sites and the relative association constant for class i, and X_{Lf} and X_{Df} are the mole fractions of free lipid and free detergent, respectively. In the limit of high amounts of detergent relative to the other components, the above equation simplifies to:

$$\bar{\nu} = \sum_{i=1}^{m} \frac{N_i K_i L_f D_t^{-1}}{1 + K_i L_f D_t^{-1}}$$

where $L_f D_t^{-1}$ is the molar ratio of free lipid to total detergent. This binding equation is similar to the one for water-soluble proteins and ligands, except that $L_f D_t^{-1}$ is the concentration variable rather than the aqueous concentration of ligand. Experimentally, the excess

detergent, which is the solvent in this system, allows the lipid/protein ratio to be varied over a wide range without the risk of protein aggregation at low lipid/protein ratios. This is an advantage over previous experiments with lipid bilayers and facilitates detection of high-affinity contact sites specific for certain lipids. Analysis of ESR data for cholate-solubilized affinity-purified bovine heart cytochrome *c* oxidase using this binding treatment, suggests that the selectivity for the negatively charged phospholipid, phosphatidylglycerol, is limited to a relatively small number of high-affinity sites on the protein surface [50].

Several aspects of the problem of lipid-protein interactions in biological membranes have been investigated by more theoretical approaches using model calculations. An example is the paper of Scott [52] who performed Monte Carlo calculations of order parameter profiles in models of lipid-protein interactions in bilayers. In these models the lipid chains are 10 carbon atoms long and interact via van der Waals forces. The chains also interact via van der Waals forces with a "perturbant", a small cylindrical model polypeptide with either a smooth or a rough surface. The results show that in all cases studied there are only slight conformational differences between the bulk chains and the chains nearest the perturbants, and Scott believes it is not possible to characterize the boundary chains as "more" or "less ordered".

Pink and MacDonald [53] report a theoretical study of the interactions between glycophorin, an integral membrane protein of the erythrocyte plasma membrane, and phospholipids in a bilayer membrane. They calculate the average value of the order parameter of a spin-labeled hydrocarbon chain in a dimyristoylphosphatidylcholine bilayer as a function of the glycophorin concentration for a temperature above the main lipid phase transition. The results indicate that between 200 and 1300 lipid molecules are affected by the large polar moiety of glycophorin dependent on its conformation. In a similar approach, the average order parameter of the fluorescent probe diphenylhexatriene was calculated and used to predict the value of the limiting anisotropy as a function of the glycophorin concentration.

The problem of determining the number (N) of lipid molecules in direct contact with integral membrane proteins in lipid bilayers and the associated problem of protein lateral distribution also has been addressed. Laidllaw and Pink [54] argue that a simple model

commonly used to determine N from ESR data obtained for lipid bilayers may lead to underestimated values of N because random protein-protein contacts, which tend to decrease the protein surface area exposed to the lipids, are not taken into account. They present an alternative model, supported by Monte-Carlo type computer simulations, and attempt to fit this model to experimental ESR data published by other workers. Unfortunately, the fit to their model heavily relies on data points obtained at low lipid-protein ratios, where it becomes troublesome to obtain preparations free of irreversibly aggregated protein. In a different approach to the problem, Mountain et al. [55] report a molecular dynamics simulation of a two-dimensional fluid mixture system designed to illustrate how short-range ordering of lipid and protein molecules might occur in membranes. In all these models the results are sensitive to the choice of input parameters. In order to adequately test these theoretical models new experimental methods must be developed to detect lipid-protein and protein-protein interactions over a much wider range of lipid-protein ratios.

3. Studies on Pure Lipid Bilayers

Koole and Hemminga [56] have analyzed the saturation properties of the in-phase and quadrature second harmonic ESR spectra of cholestane spin label in oriented lipid multibilayers in the gel phase. The in-phase and quadrature spectra exhibit different functional dependencies on the spin-lattice relaxation time T_1 of the cholestane spin label and the magnetic component of the microwave field B_1, thus enabling experimental determination of these parameters. The value of T_1 in a system of dimyristoylphosphatidylcholine with 33 mol% cholesterol increases from 1.2×10^{-6} to 3×10^{-6} S on decreasing the temperature from 20 to -10°C.

Boggs and Rangaraj [57] have investigated the phase behavior of phosphatidylcholine and phosphatidylglycerol in the presence of glycerol and polymixin by differential scanning calorimetry and ESR of fatty acid spin labels. Interdigitation of the lipid acyl chains induced by glycerol or polymixin causes a large increase in the order parameter of a fatty acid spin labeled near the terminal methyl end (16-doxylstearate). The order parameter becomes similar to that of

a fatty acid with the spin label moiety much closer to the polar head (5-doxylstearate). Thus the typical flexibility gradient found in non-interdigitated bilayers is abolished upon interdigitation and 16-doxylstearate appears useful for detection of such interdigitation. In a similar approach Boggs and Mason [58] also investigated the subgel and interdigitated gel phases formed by asymmetric phosphatidylcholines (PC).

Dynamic properties of phosphatidylcholine-cholesterol membranes in the fluid phase and water accessibility to the membranes have been studied by Kusumi et al. [59] using spin and fluorescence labeling methods. Unsaturation of the alkyl chains greatly reduces the ordering effect of cholesterol although unsaturation alone gives only minor fluidizing effects. By monitoring the dielectric environment around the nitroxide using Q-band second-derivative ESR spectra it is found that incorporation of cholesterol increases water accessibility in the hydrophilic loci of the membrane.

Lange et al. [60] have obtained ESR spectra of the 1-myristoyl-2-[6-(4,4-dimethyloxazolidine-N-oxyl)myristoyl]-*sn*-glycero-3-phosphocholine spinlabel in highly oriented, fully hydrated bilayers of dimyristoylphosphatidylcholine as a function of temperature and magnetic field orientation. The oriented spectra shown indications of slow motional components so that motional narrowing theory is not applicable to the spectral analysis. The spectra have been simulated by a line shape model incorporating trans-gauche isomerization in addition to restricted anisotropic motion of the lipid long axis. Comparison of the results with those of ^{2}H-NMR spectroscopy of dimyristoylphosphatidylcholine deuterium labeled at the same position of the *sn*-2 chain shows that most of the parameters governing chain order and dynamics are in good accord, except that the gauche population of the spin-labeled chain is considerably higher and its isomerization rate is much faster in the low-temperature phases.

Bimolecular collision rates and diffusion coefficients of lipids in biological and model membranes can be determined by comparison of experimental line shapes with simulated line shapes obtained from solutions of the exchange-coupled Bloch equations. Sachse and Marsh [61] show that fitting the normalized line heights gives an additional method of estimating the exchange frequency and thus the collision rate and diffusion coefficient. In a related paper King

and Marsh [62] measure the collision rates between spin-labeled valeric acid in water, between mixed-chain spin-labeled phosphatidylcholine in water-methanol mixtures, and between spin-labeled phosphatidylcholine monomers and micelles in water from the spin-spin broadening of the ESR spectrum. In each case the second order rate constants are consistent with a diffusion-controlled process. The much slower on-rates for association of lipid monomers with lipid bilayers compared to the monomer diffusion coefficient in water indicate that insertion into a bilayer is not a diffusion-controlled process.

In a more recent paper King et al. [63] describe an unconstrained optimization method for interpreting the concentration and temperature dependence of the linewidths of interacting nitroxide spin labels which allows determination of translational diffusion coefficients. A modified Gauss-Newton optimization procedure is used to fit an analytical expression for the total peak-to-peak, first-derivative linewidth to the concentration and temperature dependence of the ESR spectra. The temperature dependences of the contributions to the Lorentzian linewidth from both exchange and magnetic dipole-dipole interactions are inversely related via the translational diffusion coefficient. The method has been applied to the determination of the diffusion coefficients of spin-labeled phosphatidylcholine in dioleoylphosphatidylcholine over a wide temperature range.

Florine and Feigenson [64] have examined the behavior of fluorescent and spin-label probes in several fluid and gel phospholipid phases, in particular the unusual gel phase of phosphatidylserine (PS) induced by Ca^{2+} [$Ca(PS)_2$]. Anthroyloxy- and doxyl-labeled PS exhibit greatly restricted motion in $Ca(PS)_2$ whereas phosphatidylcholine (PC) and fatty acid derivatives show no apparent change in probe motion in $Ca(PS)_2$, compared to fluid lamellar lipid (PS). Experiments employing fluorescence quenching by spin-labeled PC in PS/PC in excess Ca^{2+} were used to determine the distribution of several fluorophore probes between fluid liquid-crystal and $Ca(PS)_2$ gel phases.

ESR and NMR linewidth broadening by spin labels have been used by Morrot et al. [65] to determine the overall orientation of spin-labeled analogues of cholesterol and androstanol in egg lecithin bilayers. Cholesterol analogues were found to have a single orientation in each monolayer with the acyl chain pointing towards the

center of the bilayer, while the androstanol analogue appeared to experience two opposite orientations in the same monolayer with a rapid reorientation.

Kar et al. [66] have published a detailed ESR study of spin-labeled oriented multilayers of dipalmitoylphosphatidylcholine (DPPC) at low water content. Information regarding ordering and anisotropic rotational diffusion rates was obtained via ESR line-shape analysis over the entire motional range. Cholestane (CSL) and spin-labeled DPPC have been used to probe different depths of the bilayer. Results from CSL indicate that close to the lipid-water interface the DPPC molecule is oriented approximately perpendicular to the bilayer but the lipid probes indicate that the hydrocarbon chain of DPPC may be bent away from the bilayer normal by as much as 30°, in agreement with earlier spin labeling studies [67, 68]. Electron spin echoes (ESE) were observed for the first time from oriented lipid-water multilayers. The results suggest that for detection of very slow motions ESE experiments are more sensitive to dynamics than continuous wave ESR.

Bruno et al. [69] describe a spin label ESR and ST-ESR study of the bipolar lipids extracted from *Sulfolobus solfataricus*, an extreme thermophilic archaebacterium growing at about 85°C and pH 3. These lipids are cyclic diisopranyl tetraether lipids that are quite different from the usual fatty acid lipids. The ESR spectra, obtained using positional isomers of spinlabeled stearic acid, detect phase transitions at temperatures below the physiological temperatures of *Sulfolobus solfataricus*. The head group regions in one lipid fraction exhibit a more strongly immobilized structure at 85°C than is observed for the usual monopolar lipids.

The binding of the radioprotective agent, cysteamine to dipalmitoylphosphatidylcholine (DPPC) membranes has been studied by Berleur et al. [70], using calorimetry, turbidimetry and spin labeling. A fading of the pretransition was observed by DSC and turbidimetry, suggesting that cysteamine interacts with the polar head region of DPPC bilayers. Spin-labeled fatty acids indicate increased order parameter (reduced segmental motion) of the lipid acyl chains below the gel to liquid crystal phase transition. The effect decreases above the phase transition.

Raison and Orr [71] compare various methods for detection of phase transitions in thylakoid polar lipids of chilling-sensitive plants.

ESR of spin-labeled methyl stearate, calorimetry, and fluorescence of parinaric acid all give the same temperature for initiation of the phase transition of thylakoid lipids of various plants, indicating that all three techniques present valid methods for assessing phase transitions.

Interaction between the local anesthetic, tetracaine, and egg phosphatidylcholine multibilayers has been examined by Frezatti et al. [72]. ESR spectra of a stearic acid methyl ester spin label indicate that at pH 10.5, the mobility of the spin label increases as the anesthetic: lipid ratio is increased from 0.2 to 0.6. At higher tetracaine: lipid ratios there is no increase in mobility, while above a tetracaine: lipid ratio of 1, a phase separation occurs. At pH 6.2, where the tetracaine is charged, high concentrations (e.g. 100:1 tetracaine: lipid) disrupt the bilayer to form mixed micelles. New local anesthetics (heptacaine and carbisocaine) were examined by Mazur et al. [73]. By using a spin-labeled fatty acid incorporated into phosphatidylcholine liposomes, the effects of the anesthetics on lipid order were determined from the ESR spectra. A pH dependence of the lipid disordering is observed that differs from that of the classical local anesthetics procaine and lidocaine.

Lai and Schutzbach [74] use ESR of spin-labeled stearates to investigate the effects of dolichol on the motion of lipid molecules in phospholipid membranes. Dolichols comprise a family of long-chain polyisoprenoid alcohols that are found in the membranes of most eukaryotic cells but to which few functions have yet been ascribed. The ESR spectra show that dolichol has little effect on the motion of the spin probe at carbon-5, which is already significantly motion-restricted. Dolichol reduces the motion of the spin probes at carbon-16, suggesting that dolichol molecules penetrate into the lipid core region of the lipid membranes.

4. New Lipid Spin Labels

Venkataramu et al. [75] describe the synthesis and ESR characteristics of a new stearic acid spin label substituted with deuterium in all positions and ^{15}N in the paramagnetic doxyl group. The new label displays a 5.5-fold gain in sensitivity in the ESR spectrum and a 60% decrease in linewidth compared to the unmodified analogue.

The increased sensitivity and resolution should make this label a useful one in studies employing lipid spin labels.

Acquotti et al. [76] describe a new chemical procedure for the preparation of gangliosides carrying fluorescent or paramagnetic probes on the lipid moiety. New lipid labels designed for covalent attachment to membrane proteins are discussed in Section 4.

5. *Other Studies Involving Lipid Spin Labels*

Hyde et al. [77] describe electron-electron double resonance (ELDOR) experiments on spin-labeled liposomes using a loop-gap resonator. The signal-to-noise ratio is improved 20-fold over the best that has been achieved using a bimodal cavity, permitting ELDOR experiments on spin-labeled membranes of intact cells. In a subsequent study Lai et al. [78] use ELDOR techniques employing the loop-gap resonator to measure the lateral diffusion constant, D, of lipids in the surface membrane of intact human blood platelets. Using [^{14}N], [^{15}N]-16-doxylstearate spin label pairs, D was found to be $1.0 \times 10^{-8}\,cm^2s^{-1}$ at 37°C for freshly prepared platelets but D was increased about 2.5-fold upon storage of the platelets for 3 days under routine bloodbank conditions. The increase in D may be related to loss of cholesterol from the surface membrane during storage.

Kuroda et al. [79] have performed a kinetic analysis of the fusion of hemaglutinating virus of Japan (HVJ*) containing 10 mole percent spin-labeled phosphatidylcholine with unlabeled erythrocyte membranes. The peak-height increase in the ESR spectrum due to relief of spin exchange was caused both by envelope fusion and by phospholipid exchange catalyzed by the virus-induced hemolyzate. A similar technique was used by Yamada and Ohnishi [80] to study the fusion of vesicular stomatitis virus with various cells (HELR 66, KB and human erythrocytes) and liposomes. Binding to and fusion with both cells and liposomes was enhanced at acidic pH. Binding to liposomes was dependent on the lipid head group but not on acyl chain composition, whereas cis-unsaturated acyl chains were required for efficient fusion. A cell entry mechanism is proposed in which the virus initially binds to phospholipid domains in the cell surface membrane, followed by endocytosis and fusion with the endosome upon acidification.

Keana and Pou [81] have investigated the feasibility of a nitroxide

regenerating system involving liposomes to solve the "reduction problem" when nitroxides are used as contrast enhancing agents in magnetic resonance imaging. It is shown that inclusion of the oxidant $K_3Fe(CN)_6$ entrapped in the aqueous compartment of liposomes doped with an amphiphilic nitroxide increases the duration of the nitroxide ESR signal in the presence of an external reductant.

The head group and chain length dependence of phospholipid self-assembly has been studied by King and Marsh [82] using ESR spin labeling. The critical micelle concentrations (cmc) of a series of 1-acyl-2-[4-(4,4-dimethyloxazolidine-N-oxyl)valeryl]-*sn*-glycero-3-phosphoderivatives were determined by ESR spectroscopy. The narrow, three-line ESR spectra of the rapidly tumbling monomers are easily distinguished from the spin-spin broadened spectra of the micellar aggregates allowing determination of the concentration of the two species. For phosphatidylcholine, ln[cmc] decreases linearly with the length of the *sn*-l acyl chain with a free energy of transfer of the monomer from the aqueous phase to the micelle of $-1.1RT$ per CH_2 group. For the negatively charged lipids the cmc's decrease by 1–2 orders of magnitude on increasing the ionic strength from 0 to 2.0 M NaCl, while the salt dependence is considerably less for the zwitterionic lipids. Thus the polar head group can have a marked effect on the energetics of self-assembly and correspondingly on the kinetics of lipid transfer between vesicles.

Coan [83] has studied the distribution of the lipophilic spin probe, 5-doxyl stearate, between the inner and outer halves of sarcoplasmic reticulum (SR) membranes by titration with the spin broadening agent Ni·EDTA. It is found that the spin probe distribution between outer and inner halves is 35:65, whereas the distribution in small sonicated vesicles prepared from SR lipids is 60:40, consistent with the vesicle geometry. It is concluded that the observed asymmetry in distribution in the SR membranes is due to the presence of SR proteins. Changes in the conformation of one of the SR membrane proteins, the ATPase, induced by Ca^{2+} and adenyl-5′-yl imidodiphosphate·Mg binding did not affect the spin probe distribution but phosphorylation of the enzyme gave some protection of probe signal from the spectral broadening reagent. Ascorbate reduction indicated that a small fraction of the spin probes, and possibly of the SR lipids, may be occluded following phosphorylation.

Membrane lipids of the general fatty acid auxotrophic bacterium *Butyrivibrio* S2 have been characterized by Hauser et al. [84] employing differential scanning calorimetry and ESR spectroscopy. Lipid mobility, as determined by ESR, is low compared to mammalian plasma membranes but is comparable to that of other bacterial membranes. Membranes of the organism grown with saturated fatty acids of defined hydrocarbon chain length undergo a broad endothermic phase transition with the end-point temperature approximately coinciding with the minimum temperature supporting growth. ESR and calorimetry evidence together suggest that, at the growth temperature, the plasma membrane of *Butyrivibrio* S2 is in the liquid-crystalline state. The ESR order parameter of cell membranes of this organism was found to be similar regardless of whether it was grown on myristic, palmitic or stearic acid. *Butyrivibrio* S2 thus has a mechanism enabling it to maintain membrane packing and fluidity at fairly constant level, although it cannot alter either the chain length or unsaturation of membrane fatty acids.

Herring et al. [85] examine the fluidity of lipids (i.e., segmental motion of the spin label, 5-doxyl stearic acid) in membrane preparations from a mutant of *Escherichia coli* that is resistant to the uncoupler carbonyl cyanide m-chlorophenylhydrazone (CCCP). Growth in the presence of CCCP results in a decrease in membrane lipid fluidity but the fluidity of lipids extracted from these membranes is increased, concomittant with an increase in the proportion of unsaturated fatty acids. The lower fluidity of the lipids in these membranes may be a consequence of an increase in the ratio of protein to phospholipid.

Cation-induced changes in the membrane fluidity of isolated corn mitochondria have been investigated by Cooke et al. [86] using stearic acid spin labels with the nitroxide group close to the polar head and close to the methyl end (5- and 16-doxyl stearic acids, respectively). Addition of Ca^{2+} or La^{3+} results in a decrease in the motion of the labels within the mitochondrial membranes. A comparison of the temperature-induced changes in label motion with those induced by Ca^{2+} indicates that the 16-doxyl stearic acid is more sensitive to Ca^{2+}-induced changes than is 5-doxyl stearic acid. However, one must keep in mind that the segmental motion increases along the fatty acid chains. Thus even before the addition of divalent cations, there is less motion near the surface because of the

interaction of the polar head groups. It is, therefore, not unreasonable to expect a larger effect with 16-doxyl stearic acid than for 5-doxyl stearic acid.

The effect of the inhibitory neurotransmitter γ-aminobutyric acid (GABA) on inhalation anesthetic-induced membrane fluidization is described by Rosenberg and Alila [87]. The anesthetic enflurane at 2 mM fluidizes the lipids of synaptic plasma membranes of whole rat brain or of the straitum, synaptic mitochondrial membranes, and dipalmitoyl lecithin vesicles, both in the polar head and core regions. GABA, added prior to the anesthetic, causes a dose-related inhibition of the fluidization. This restorative effect also occurs in lipid vesicles and is not prevented by GABA antagonists, and is therefore probably not related to receptor mechanisms.

Utsumi et al. [88] use spin-labeled stearic acids with the doxyl group attached at the 5- or 12-carbon atom and the corresponding methyl esters to estimate the membrane fluidity of rat liver microsomal membranes after oral administration of carbon tetrachloride. The results suggest that CCl_4 or its metabolites increase membrane fluidity primarily at hydrophobic regions rather than at the surface layer. Alterations in the hepatocyte plasma membrane following CCl_4 poisoning are reported by James et al. [89]. Freeze-fracture electron microscopy revealed an increase in the individual mean gap junction size in rats treated with CCl_4. ESR spectra of hepatocytes incubated with 12-doxylstearic acid revealed that although the order parameter was not altered by CCl_4 exposure in vivo, the rotational coefficient was significantly smaller. The data suggest that CCl_4 may interact with the membrane in a manner which alters membrane lipid fluidity thus influencing gap junction particle segregation and aggregation.

Fluidity of the plasma membrane of the bloodstream form of *Trypanosoma brucei* has been examined with ESR and fluorescence spectroscopy by Munske et al. [90]. The temperature dependence of the order parameter for 5-doxyl stearate and of 8-anilino-1-naphtalene sulfonate polarization indicates phase alterations near 30°C. Proteolysis of the surface glycoprotein with trypsin increases fluidity but treatment with human serum, which is trypanocidal, has no effect.

Hongo et al. [91] report a spin label study of the effect of ticlopidine on platelets. In mammals, ticlopidine inhibits ex vivo platelet aggregation through an as yet unknown mechanism. This

study shows that ticlopidine, orally administered to rats, increases both the order parameter and the apparent rotational correlation times of 5- and 16-doxylstearic acid spin probes incorporated into platelet membranes. Thus the inhibitory action of the drug or its metabolites is accompanied by changes in the segmental motion of the lipids.

Chattopadhyay and London [92] describe an interesting method to determine the depth of penetration of a wide variety of fluorescent molecules embedded in membranes utilizing fluorescence quenching by spin-labeled phospholipids. The method involves determination of the parallax in the apparent localization of fluorophores detected when quenching by phospholipids spin labeled at different positions along the acyl chain is compared. The method has been applied to lipids covalently labeled with the fluorophor 7-nitro-2,1,3-benzoxadiazol-4-yl (NBD). Spin-labeled lipids with the nitroxide moiety located at three different depths were used to quench the fluorecence. Quenching experiments show that, as expected, the NBD group of head-group-labeled phosphatidylethanolamine is at the lipid-water interface, while the NBD label on the "tail" of cholesterol is deeply buried. This method could prove generally useful in determining the localization of fluorophores, for example tryptophan residues of membrane proteins, in biological membranes.

Taraschi et al. [93] compare ethanol-induced structural perturbations in the membranes of rat hepatic microsomes measured with the spin probe 12-doxyl stearic acid with those assayed with the phospholipid spin probes 1-palmitoyl-2-(12-doxylstearoyl) phosphatidylcholine, -phosphatidylethanolamine and -phosphatidic acid. Both the fatty acid and the phospholipid spin probes show increased disordering (increased motion) of the membrane lipids with the in vitro addition of increasing amounts of ethanol but the effect noted with the phospholipid spin probes is quantitatively less. Both types of probes show that microsomes obtained from the livers of chronically alcohol-intoxicated animals are resistant to the disordering effects of ethanol in vitro. These results suggest that fatty acid spin probes are qualitatively valid for measuring membrane perturbations and that ethanol affects microsomal phospholipids regardless of head group structure.

Membrane mechanisms of ethanol tolerance in *Saccharomyces cerevisiae* have been examined by Curtain et al. [94, 95]. Employing distearoylphosphatidylcholine with a nitroxide group attached to

either the 5- or 16-carbon atom of one of the acyl chains it is shown that increasing concentrations of ethanol have a much more fluidizing effect on the yeast plasma membranes than on phospholipid vesicles with the same lipid composition. The fluidization is interpreted as being due to the displacement of annular lipids from the integral membrane proteins.

The lipid phase of transverse tubule membrane of skeletal muscle has been probed with fatty acid spin labels by Hidalgo [96]. The motion of the probe increases as the distance between the spin label and the polar head increases, as in other membranes. However, the value of the order parameter for a fatty acid spin label containing the label attached to the 5-carbon atom is higher than reported for other mammalian membranes. Order parameters for spin labels containing the label nearer to the center of the bilayer are closer to the values reported for other mammalian membranes. The lipid phase of the transverse tubule membrane is thus less fluid, at least as far as the motion of the acyl chain segments near the polar head groups is concerned, than that of other membranes, sarcoplasmic reticulum membrane in particular. This may be related to the high cholesterol content of transverse tubules.

The influence of ganglioside insertion into microsomal membranes from calf brain on the rate of ganglioside degradation by membrane-bound sialidase has been investigated by Scheel et al. [97]. Micelles of exogenously added ganglioside G_{D1a} bind to microsomal membranes in two steps: fast adsorption, followed by slower uptake (insertion). The product of the sialidase degradation, ganglioside G_{M1}, is found in the ganglioside pool taken up by the membranes only. ESR studies using a spin-labeled analogue of ganglioside G_{D1a} suggest that exogenously added ganglioside inserts into the membranes before it is recognized as substrate by the sialidase. The effect of pH, ionic strength and fluidizing agents on the rate of insertion should, therefore, be taken into account when the influence of these parameters on the rate of the sialidase reaction is studied.

Herring et al. [98] and Burt et al. [99] have studied the nature of the membranolytic interaction between crystals of monosodium urate monohydrate (MSUM) and membranes using ESR. MSUM crystals produce the inflammatory reaction of acute gouty arthritis by a mechanism which is believed to involve interactions between

lysosomal membranes and crystals taken up by phagocytosis, resulting in membrane rupture. Two spin probe molecules, negatively charged 5-doxyl stearic acid and positively charged N,N-dimethyl-N-dodecyltempoylammonium bromide (CAT_{12}), were incorporated into intact human erythrocytes and incubated with MSUM crystals. The stearic acid spin label is located entirely in the membrane and the (CAT_{12}) label partitions between the membrane and aqueous phase. Incubation with MSUM increases the apparent fluidity of the stearic acid spin label, and reduces the fraction of membrane-bound CAT_{12}. These observations are interpreted as being caused by electrostatic interactions between the negatively charged MSUM crystals and the charged spin probes.

B. Non-Lipid Spin Labels

The chemical structures of some examples of small, non-lipid spin probes used in ESR studies of biological membranes are shown in Figure 4. The top row (structures a-d) shows freely diffusing spin labels which are all derived from TEMPO (a). Structures e and f are

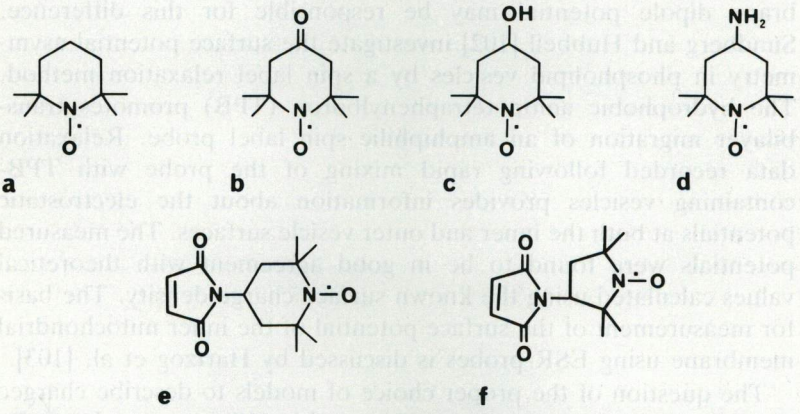

FIGURE 4 Chemical structures of small spin probes employed in spin labeling studies of biological membranes: a, TEMPO; b, TEMPONE; c, TEMPOL; d, TEMPAMINE. Structures e and f are maleimide spin labels that attach primarily to sulfhydryl groups in proteins and are commonly used for labeling of membrane proteins to investigate their dynamic behavior.

maleimide spin labels that are used for covalent attachment of nitroxides to membrane proteins (see section 4).

Earnest et al. [100] have examined the effect of the protein surface potential on the binding of a spin-labeled quaternary amine local anesthetic to the nicotinic acetylcholine receptor from *Torpedo californica* reconstituted into bilayers of phosphatidylcholine. ESR of membranes labeled with the spin-labeled local anesthetic, previously shown to block the receptor-ion channel function, shows a spectral component corresponding to the spin label immobilized by protein. With the receptor reconstituted into the zwitterionic phosphatidylcholine, which is assumed to have no surface potential of its own, it is demonstrate that the spin-labeled local anesthetic binds to the acetylcholine receptor as a function of the surface potential on the protein.

Flewelling and Hubbell [101] describe the synthesis of a spin-labeled analogue of the hydrophobic ion tetraphenylphosphonium (TPP^+). The thermodynamic properties of the interaction of TPP^+ with egg phosphatidylcholine vesicles were studied by equilibrium dialysis and ESR spin labeling. It was found that the binding is entropy-driven with a positive (repulsive) enthalpy of binding, while the binding enthalpy is negative for hydrophobic anions. The membrane dipole potential may be responsible for this difference. Sundberg and Hubbell [102] investigate the surface potential asymmetry in phospholipid vesicles by a spin label relaxation method. The hydrophobic anion tetraphenylboron (TPB) promotes transbilayer migration of an amphiphilic spin label probe. Relaxation data recorded following rapid mixing of the probe with TPB-containing vesicles provides information about the electrostatic potentials at both the inner and outer vesicle surfaces. The measured potentials were found to be in good agreement with theoretical values calculated using the known surface charge density. The basis for measurement of the surface potential of the inner mitochondrial membrane using ESR probes is discussed by Hartzog et al. [103].

The question of the proper choice of models to describe charged phospholipid bilayers has been addressed by Winiski et al. [104]. By measuring the adsorption of a fluorescent monovalent anion and a paramagnetic divalent cation to both positive and negative membranes, predictions of a discreteness-of-charge theory were tested and compared to those of the familiar Gouy-Chapman-Stern (GCS) theory which assumes that the charge is uniformly smeared over the

surface. All experimental results, including those obtained for membranes in the gel phase, are in closer agreement with the predictions of the GCS theory than with a discreteness-of-charge model. A similar conclusion is reached by Hartsel & Cafiso [105] based on measurements of the surface potentials of model membrane systems using a new series of negatively charged, spin-labeled alkylsulfonate probes (see Figure 5), in conjunction with positively-charged spin labels.

Keana et al. [106, 107] describe the preparation of a new series of 9,10-disubstituted-2-anthracenyl *tert*-butyl nitroxides aimed at developing a nitroxide based ESR probe for singlet oxygen. One of the anthracenyl nitroxides reacts with singlet oxygen to give an endoperoxide both in organic solvent and when the nitroxide is incorporated in the bilayers of dimyristoylphosphatidylcholine vesicles. The reaction is sufficiently rapid and the resulting spectral changes are sufficiently characteristic for the anthracenyl nitroxide to serve as an ESR-based probe for singlet oxygen.

The orientation and mobility of a square-planar copper complex has been investigated by Subczynski et al. [108]. By using the spin label, TEMPO (2,2,6,6-tetramethylpiperidine-1-oxyl), the copper, complex, CuKTSM2, a derivative of a potent antitumor drug, was found to partition favorably into dimyristoylphosphatidylcholine vesicles. Saturation recovery ESR techniques were used to measure the effect of bimolecular collisions between Cu complexes and stearic acid spin labels on the spin-lattice relaxation time of the nitroxide moiety. The translational diffusion constant of the complex was found to be about ten times greater than that of the lipids in the fluid phase.

Erriu et al. [109] use differential scanning calorimetry in combination with ESR of the spin probe, TEMPO, to examine the phase

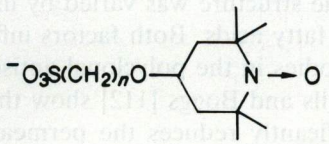

FIGURE 5 Chemical structure of the alkylsulfonate nitroxides described by Hartsel & Cafiso [105].

behavior of γ-irradiated lipid membranes. The sharp variation in the fraction of TEMPO bound to the lipid phase that occurs at the main transition temperature is much less sharp with irradiated samples. At greater irradiation doses the products of irradiation lead to the formation of phase-separated domains.

Boggs and coworkers [110, 111, 112] have published a series of studies describing the effect of various factors on the permeability of lipid vesicles using the water-soluble spin label TEMPOcholine chloride. At the high concentrations of TEMPOcholine trapped in these vesicles, exchange broadening markedly reduces the height of the ESR signal. As the TEMPOcholine is diluted into the surrounding aqueous environment due to its leakage from the vesicles, the signal height is increased, providing a measure of permeability. The ability of charge isomers of myelin basic protein (BP) to increase the permeability of multilamellar vesicles composed of phosphatidylserine/phosphatidylcholine and sphingomyelin/cholesterol/phosphatidic acid was measured by monitoring the release of TEMPOcholine [110]. The increase in vesicle permeability caused by BP was taken as a measure of the degree of perturbation of the bilayer by the protein. All classes of charge isomers of BP were more effective at increasing vesicle permeability than polylysine, but a phosphorylated isomer of BP was less effective than the other isomers. Small changes in protein charge can apparently influence the interaction of BP with the lipid bilayer. The same technique was used to measure the influence of lipid environment and ceramide composition on antibody recognition of cerebroside sulfate in liposomes [111]. The surface expression of the glycolipid, i.e., the reactivity with antibody, was determined by measuring complement-mediated lysis of the TEMPOcholine-containing vesicles. The lipid environment was varied by using phosphatidylcholine of varying chain length in a mixed vesicle with cholesterol and cerebroside sulfate. The ceramide structure was varied by using synthetic forms containing different fatty acids. Both factors influenced the binding of the various antibodies in the polyclonal antiserum. In a separate study, Kurantsin-Mills and Boggs [112] show that a low concentration of serum significantly reduces the permeability of liposomes, whereas serum is generally regarded to increase permeability. It is found that the effect of serum on liposome permeability depends on the compound entrapped as well as the type of lipid used.

Lagercrantz et al. [113] describe the effect of membrane-active drugs on the entrapping of the spin label TEMPOcholine into human erythrocytes that occurs when the cells spontaneously reseal after being subjected to hyposmolar stress. Chlorpromazine, trifluoperazine, nicardipine, amperozide and haloperidol give rise to a dose-dependent decrease of the entrapping of TEMPOcholine. It is suggested that these substances exert their action on the resealing process by interacting with the calmodulin system.

Gwozdzinski [114, 115, 116] describes experiments on the effect of ionizing radiation and thiol-reactive reagents on the permeability of porcine and fish (carp) erythrocytes to charged and uncharged small water soluble spin labels. Both factors affect the erythrocyte permeability dependent on the nature of the spin label. For example, irradiation of porcine erythrocytes increases the permeability of the spin probes TEMPO and TEMPOL. The thiol reagents N-ethylmaleimide and p-chloro-mercuribenzoate decrease the permeablity of these spin probes and the effect is greater with irridiated erythrocytes.

Several papers report use of non-lipid spin probes to determine internal cellular volumes. The method consists of incubating cells or vesicles with a permeable spin probe, e.g., TEMPONE (2,2,6,6-tetramethyl-4-oxopiperidine-N-oxyl), and a non-permeable quenching agent, e.g., $Na_2MnEDTA$ or other complexes containing transition metals. The quenching agent broadens the external spin probe signal so that the relative signals before and after addition of the quenching agent give the internal volume as a fraction of the total aqueous volume. An example is the paper of Ball et al. [117], who use this method to measure intrathylakoid aqueous volumes to study ionic permeability properties of thylakoid membranes and the shrinking and swelling of thylakoids exposed to salt solutions. Essentially the same technique is used by Anzyr et al. [118] to measure the intracellular solvent volume of sclerotic cells of the fungus *Claviceps purpurea*. These authors also describe a technique to determine the total cellular volume (the volume including the cell walls) using a charged impermeable spin probe. Lomax et al. [119] use paramagnetic spin probes to quantitate both apparent vesicle volume and the transmembrane pH gradient of closed and pH-tight membrane vesicles prepared from hypocotyls of *Cucurbita pepo* (zucchini) seedlings. A weak acid nitroxide probe, TEMPACID

(2,2,6,6-tetramethyl-4-carboxylpiperidine-N-oxyl), which partitions preferentially into basic environments, was used to estimate $\triangle$pH. These measurements were used to study uptake of the plant growth hormone indole-3-acetic acid into the vesicles. It is concluded that uptake is an active process driven by the pH gradient. Measurements using nitroxide spin probes to determine internal volumes, proton gradients, and electrical potentials also are described by Mehlhorn et al. [120].

IV. SPIN LABELS COVALENTLY ATTACHED TO MEMBRANE PROTEINS

The period covered in this review has seen a number of publications in which new spin labels for covalent attachment to membrane proteins are described. Goals in designing these covalent spin labels have been two-fold: (i) To obtain information on lipid dynamics in the region of contact between membrane proteins and lipids; and (ii) To obtain information on rotational and translational mobility of membrane proteins in native membranes and lipid bilayers.

Griffith et al. [121] describe the synthesis of a phospholipid photo-spin label, an analogue of phosphatidylethanolamine (PE) with tritium labeled 14-proxylstearic acid esterified at the *sn*-2 position and a nitroaryl azido group incorporated in the polar head (Figure 6). Reconstitution of bovine heart cytochrome *c* oxidase with the photo-spin label, followed by photolysis, results in very efficient (50%) covalent attachment of the label to the protein. Line shape analysis of ESR spectra of the labeled protein as a function of the total lipid/protein ratio in the samples provides additional support for the notion that the fraction of lipids normally in contact with the protein, and not an aggregation artifact, accounts for the motion-restricted component observed in membranes.

From the same laboratory, McMillen et al. [122] describe the synthesis, characterization and application of a new series of amine-specific phospholipid analogues based on the benzaldehyde reactive group (Figure 7). All of these rèagents are either radiolabeled or spin-labeled or both, thus permitting identification of protein regions

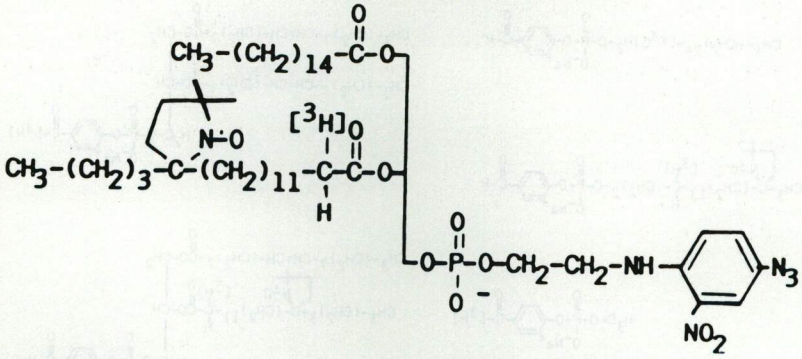

FIGURE 6 Chemical structure of the phospholipid photo-spin label described by Griffith et al. [121].

in contact with the phospholipid head groups as well as investigation of the dynamic behaviour of lipid acyl chains at the protein-lipid interface. Reaction of the lipid-benzaldehydes with cytochrome *c* oxidase results in preferential labeling of some of the polypeptide components of this integral membrane protein. ESR spectra of a spin-labeled lipid benzaldehyde covalently attached to cytochrome *c* oxidase exhibit a large motion-restricted component with a line shape and splitting similar to that of freely diffusable lipid spin labels, providing independent evidence that coupling of the lipid benzaldehydes occurs at the protein-lipid interface. Recently, Kuppe et al. [123] extended this approach with the synthesis of a cardiolipin benzaldehyde label.

The intrinsic membrane protein rhodopsin reconstituted into lipid bilayers continues to be a system well-suited for investigation of the effect of the lipid environment on the conformation and function of a membrane protein. Baldwin and Hubbell [124] show by ESR using a novel disulfide spin label (Figure 8) that is covalently linked to rhodopsin reconstituted in dimyristoylphosphatidylcholine, that the apparent arrest of the protein at the metarhodopsin I stage is probably not due to aggregation of the protein in this un-physiological lipid environment. Spin-labeling of approximately two sulfhydryl groups per opsin molecule demonstrates that the spin label is in a relatively mobile environment. Only after hydrolysis of about

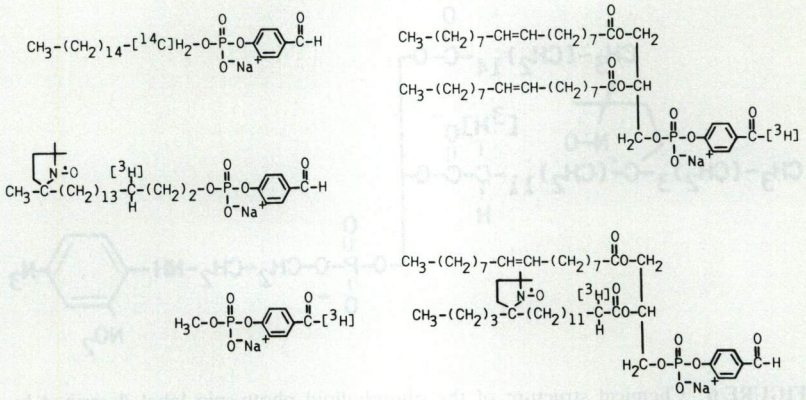

FIGURE 7 Chemical structures of the phospholipid benzaldehyde labels described by McMillen et al. [122]. The molecule on the lower left is a water-soluble benzaldehyde used for comparison with the long-chain membrane-soluble lipid benzaldehydes.

75% of the lipids by phospholipase C, which produces extensive two-dimensional lateral aggregates of rhodopsin molecules, is a highly motion-restricted ESR line shape observed.

Beth and coworkers [125, 126] have studied the dynamics and interactions of the anion channel (band 3) in intact human erythrocytes employing a new membrane-impermeant, homobifunctional spin-labeling reagent, bis-(sulfo-N-succinimidyl) doxyl-2-spiro-4′-pimelate (BSSDP) (Figure 9). BSSDP reacts with high specificity with the extracytoplasmic domain of band 3. The conventional (linear) ESR spectrum of intact BSSDP-labeled erythrocytes shows that the spin label is immobilized on the protein and spatially isolated. ST-ESR of the same sample indicates that the anion channel (band 3) in intact erythrocytes exhibits rotational dynamics with an effective correlation time in the range of 0.1−1 ms at 20°C, consistent with a strong interaction of this protein with the erythrocyte cytoskeleton. Upon lysis of the cells these interactions are significantly disrupted as indicated by an increase in rotational mobility.

Other groups have employed previously described covalent spin

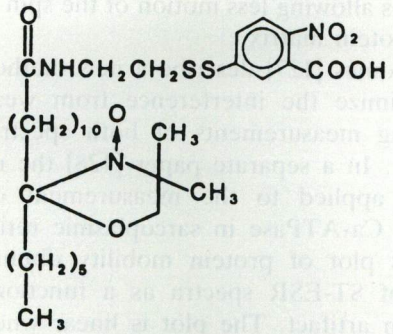

FIGURE 8 Chemical structure of the disulfide spin label described by Baldwin and Hubbell [124].

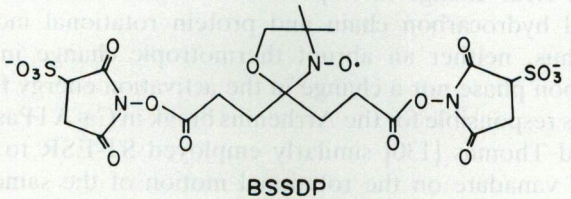

FIGURE 9 Chemical structure of the bifunctional spin-labeling reagent BSSDP described by Beth et al. [125, 126].

labels, in particular maleimide spin labels (Figure 4), to study the rotational dynamics of membrane proteins. A problem in determining the rotational mobility of membrane proteins is that the presence of small amounts of weakly immobilized probes can result in large systematic errors in the (effective) rotational correlation times determined from ST-ESR spectra. This problem is probably more severe when monofunctional rather than bifunctional covalent spin labels are employed. The latter can be expected to be more

tightly bound thus allowing less motion of the spin label moiety with respect to the protein matrix.

Squier and Thomas [127] describe a new methodology based on ST-ESR to minimize the interference from weakly immobilized probes combining measurements of both spectral lineshape and spectral intensity. In a separate paper [128] the new experimental methodology is applied to the measurement of the rotational dynamics of the Ca-ATPase in sarcoplasmic reticulum. The non-linear Arrhenius plot of protein mobility obtained through line shape analysis of ST-ESR spectra as a function of temperature appears to be an artifact. The plot is linear when intensity parameters (spectral integral) are used or when the spectra are corrected by subtracting out the weakly immobilized component prior to line shape analysis. The linear Arrhenius plot is consistent with the theory for the hydrodynamic properties of a membrane protein of constant size and shape in a fluid bilayer. Interestingly, Bigelow et al. [129] show that an Arrhenius plot of the Ca-ATPase *activity* still exhibits a clear change in slope at 20°C, while Arrhenius plots of both lipid hydrocarbon chain and protein rotational mobility are linear. Thus, neither an abrupt thermotropic change in the lipid hydrocarbon phase nor a change in the activation energy for protein mobility is responsible for the Arrhenius break in Ca-ATPase activity. Lewis and Thomas [130] similarly employed ST-ESR to study the effects of vanadate on the rotational motion of the same enzyme. It is shown that decavanadate substantially immobilizes the spin-labeled protein indicating protein-protein association whereas monovanadate inhibits the enzyme without a change in protein mobility.

Fajer et al. [131] use saturation-recovery electron paramagnetic resonance (SR-EPR), a time-resolved saturation transfer ESR technique, to measure directly the microsecond rotational diffusion of spin-labeled proteins. This method uses an intense microwave pulse to saturate a spin population having a narrow distribution of orientations with respect to the magnetic field. The recovery phase is then analyzed. The saturation recoveries of spin-labeled hemoglobin tumbling in medias of known viscosities were measured as a function of rotational correlation time and pulse duration and rotational correlation values estimated from the initial phase of the recovery were in good agreement with theory. The results demonstrate that

SR-EPR is applicable to the study of the motion of spin-labeled proteins, and, in principle, has advantages compared to the established optical techniques.

Hornblow et al. [132] discuss the use of changes in the ratio of weakly to strongly immobilized ESR signals of erythrocyte membranes nonspecifically labeled with the maleimide spin label to monitor drug-erythrocyte interactions. Factors such as label/protein ratio, labeling time, temperature, and time after labeling affect the ratio of weakly to strongly immobilized signals. Thus it is necessary that all of these factors be rigorously kept constant when this method is used to monitor drug binding to erythrocytes. Bartosz and Gaczynska [133] observe that the ratio of low-field amplitudes of weakly and strongly immobilized signals in ESR spectra of a maleimide spin label bound to erythrocyte membranes increases progressively during incubation at 37°C. The authors believe this is due to self-digestion of membrane proteins by endogenous proteases and this suggests a need for careful interpretation of data from spin-labeled membrane proteins, especially when longer incubations are involved.

Freeman et al. [134] describe covalent labeling of cultured thoracic aorta endothelial cells with a maleimide spin label under conditions where cell viability is not affected. The ratio of the peak amplitudes of the weakly and more strongly immobilized species in the ESR spectrum was irreversibly elevated following incubation with a superoxide-generating system, indicating increased membrane fluidity. The endothelial cell membranes appear useful as a model for assessing radical-mediated cell damage.

Effects of sodium valproate (VPA), a major antiepileptic drug used in the treatment of generalized epileptic seizures, on mitochondrial membranes have been studied by Rumbach et al. [135]. VPA-treated mitochondria spin-labeled with a maleimide spin label show a reduction in the ratio of weakly to more strongly immobilized signal compared to control mitochondria. Spectra of mitochondrial lipids spin-labeled with 5-doxyl stearic methyl ester show, however, that VPA has no significant effect on the lipid order parameters. These observations relate to the therapeutic action of VPA and its effect on membranes. Fujimura et al. [136] study the effect of glutaraldehyde on the protein and lipid environment of mitochondria using both covalent (maleimides) and non-covalent

(stearic acid) spin labels. The data indicate that glutaraldehyde reduces the motion of the protein-bound maleimide spin label. Some restriction of lipid mobility near the headgroup region is also observed.

Molecular properties of cetiedil, an antisickling agent and a vascular smooth muscle relaxant, and its interactions with erythrocyte membranes have been studied by Narasimhan and Fung [137]. Large amounts of cetiedil associate with erythrocyte ghosts and ESR experiments utilizing maleimide and fatty acid spin labels show that the motion of both protein and lipid is affected. Binding of cetiedil is not affected by removal of the spectrin and actin network. Band 3 molecules are implicated in the interaction of cetiedil with the erythrocyte membrane.

Gwak et al. [138] have studied the interaction between mitochondrial succinate-ubiquinone and ubiquinol-cytochrom *c* reductases by differential scanning calorimetry (DSC) and ST-ESR. The DSC results suggest a specific interaction between the reductases in the membrane. This idea is supported by ST-ESR data showing that the rotational correlation time of maleimide spin-labeled ubiquinol-cytochrome *c* reductase is increased when mixed with succinate-ubiquinone reductase prior to embedding in phospholipid vesicles.

Dalton et al. [139] estimate the distance of separation of the reactive sulfhydryl of D-β-hydroxybutyrate dehydrogenase from the bilayer surface. The enzyme, derivatized with the maleimide spin label in the presence of the cofactor NAD^+, was inserted into phospholipid vesicles and titrated with the spin probes Mn^{2+} or Gd^{3+} until the ESR spectrum was reduced in amplitude to its limiting value. From this limiting amplitude the radial distance of closest approach of the paramagnetic ions to the spin label on the enzyme was estimated to be 17 Å. Since the nitroxide moiety of the maleimide spin label is 8 Å from the sulfhydryl group, the two limiting distances of immersion of the reactive sulfhydryl within the bilayer are 9 and 25 Å, where the shorter distance is considered more compatible with facile access of the coenzyme to the active site of the enzyme.

Sharom and Ross [140] describe covalent spin labeling of sialic acid and galactose residues on the lymphocyte plasma membrane. Employing specific activation of sugars with periodate or galactose oxidase, followed by reductive amination with TEMPAMINE, spin label probes are coupled to glycoproteins or glycolipids at the

lymphocyte cell surface. Binding of several lectins to these preparations produces significant immobilization of cell surface oligosaccharides while others have no effect, dependent on the sugar specificity of the lectin. Binding of lectins to the lymphocyte cell surface thus seems to have differential effects on the dynamic states of glycolipids and glycoproteins within the glycocalyx. By a similar procedure, Farmer and Butterfield [141] use perdeuterated, ^{15}N-TEMPAMINE spin label to selectively label cell surface sialic acid of human erythrocyte membranes. This covalent spin label shows increased sensitivity and decreased data scatter compared to its protonated ^{14}N-analogue.

Kunicki et al. [142] describe a general method for the production of nitroxide-labeled antibodies. Fab′ fragments are generated from murine monoclonal antibodies and a free sulfhydryl group is introduced in the carboxyl-terminal region of the molecule through reduction of a disulfide bridge with cysteine. The sulfhydryl group is then alkylated with maleimide spin label, thereby generating spin-labeled Fab′ fragments. Two monoclonal antibodies, each specific for a different membrane glycoprotein of human platelets were tested. The spin-labeled Fab′ fragments retain their ability to bind to these glycoproteins in membranes of intact platelets, indicating that the Fab′ spin probes can be used to monitor integral membrane protein mobility. However, the ESR spectra indicate considerable motion of the spin label coupled to the Fab′ fragment. Modification of this approach may be needed for studies where the motion of the Fab′-antigen complex is of interest.

V. SUMMARY

Spin labeling is now an established spectroscopic technique for studies of membrane structure and function. What has occurred during the past five years is a broadening range of applications, the addition of some new spin labels, and the refinement of some methods of analysis. Several different strategies have evolved for answering specific biological questions. One of the most popular approaches is to use a freely-diffusing spin label that mimics a

naturally-occuring component of the membrane. For example, a lipid analog containing the spin label can be introduced into the system of interest. Changes in segmental motion of lipid chains with temperature, change of composition of the membrane, or upon addition of other lipid soluble molecules are detected through line shape changes in the ESR spectra and correlated where possible with membrane function (membrane receptor, ion channel, or enzymatic activity). An important variation on this approach is the study of lipid-protein interactions using freely diffusable spin labeled lipids. Quite fortuitously, the time scale of the ESR experiment is much better that that of NMR and most other spectroscopic techniques for the study of lipid-protein interactions. Lipid in contact with protein exhibits a motion-restricted spectral component readily distinguishable from the bilayer line shape. In most cases the lipid exchange on and off the protein surface does not average this information. From the ratios of the two components, the equilibrium constant for protein binding by one lipid can be determined relative to a reference lipid. The presence of the spin label has been shown not to perturb the equilibrium. Considerable progress has been made in characterizing the relative binding or occupancy of sites around the hydrophobic protein surface using this approach. Many questions remain including two central ones: Why do membranes contain a diversity of lipids and what is the significance of the asymmetry in lipid composition? The challenge experimentally is to extend the range of lipid/protein ratios that can be examined.

A very different approach is to covalently attach a spin label to proteins. New labels have been added in recent years and the technique of saturation transfer ESR has extended the time scale to the submillisecond region. In these studies the biochemistry is assuming an increasingly important role. The results are only as good as the specificity and site of labeling, so that in many cases the protein of interest is isolated, labeled, and then reintroduced into the biological system.

Other types of information used in studies of biological membranes include exchange interactions between spin labels (to detect clustering of labels) and dipolar effects (to determine the orientation of probes). Not all spin labels are inert. The susceptability of some spin labels to chemical reduction continues to be useful in determining the kinetics of transbilayer movement. Another application, outside of the scope of this review, is the use of spin labels as

contrast enhancers in magnetic resonance imaging. With the increasing sensitivity of NMR, it is also likely that applications in membrane research will utilize the effects of spin labels on the relaxation times of nuclei in NMR.

The future appears to be bright for spin labeling as well as other spectroscopic labeling methods. With gene cloning and expression, more proteins, present in minute quantities under physiological conditions, will become available for biophysical studies. Site directed mutagenesis, combined with advances in organic chemistry to produce new spin labels, and advances in magnetic resonance spectroscopy will make it possible to answer increasingly sophisticated questions about the function and regulation of cellular processes.

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