Selective Protein–Lipid Interactions at Membrane Surfaces: A Deuterium and Phosphorus Nuclear Magnetic Resonance Study of the Association of Myelin Basic Protein with the Bilayer Head Groups of Dimyristoylphosphatidylcholine and Dimyristoylphosphatidylglycerol†

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ABSTRACT: Basic protein from bovine spinal cord was reconstituted into bilayers of 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine, 1,2-dimyristoyl-*sn*-glycero-3-phospho-*rac*-glycerol, and equimolar mixtures of both phospholipids at various protein concentrations up to 58 wt %. The lipids were selectively deuterated at all positions in their polar head groups, the motional rate and amplitude of which were characterized by deuterium and phosphorus-31 nuclear magnetic resonance (NMR). Basic protein, which binds peripherally to membranes, did not perturb any part of the head groups in phosphatidylcholine bilayers in such a way as to induce any significant changes in the NMR spectra. However, basic protein induced large effects on the phosphorus-31 and the deuterium NMR spectra of bilayers of dimyristoylphosphatidylglycerol. When compared to those of pure phosphatidylglycerol bilayers at 35 °C, the measured quadrupole splittings decreased by 36%, 28%, and 50% for the α-CD_2, β-CD, and γ-CD_2 glycerol segments, respectively, and the phosphorus-31 chemical shift anisotropy (CSA) was reduced by 34% from −39 to −25 ppm by the addition of 50 wt % of basic protein. Deuterium spin–lattice relaxation times T_1 for the β-CD and the γ-CD_2 segments decreased only slightly after the addition of basic protein. These results indicate that basic protein interacts with the acidic polar head group of phosphatidylglycerol, affecting its orientation or amplitude of motion, as well as its rate of high-frequency segmental motions. Basic protein was also reconstituted into mixed bilayers containing equimolar amounts of labeled and unlabeled phospholipid. When the head group of phosphatidylglycerol contained the label, the quadrupole splittings, spin–lattice relaxation times T_1, and phosphorus-31 CSA values were sensitive to increasing amounts of protein up to 50 wt %, the magnitude of the induced changes being very similar to those measured for phosphatidylglycerol bilayers devoid of phosphatidylcholine. However, the major perturbation of the phosphatidylglycerol head group motion in these mixed bilayers was due not to the protein but to the phosphatidylcholine lipids. For mixed bilayers in which the phosphatidylcholine was labeled, the experimental parameters showed little change when up to 58 wt % of basic protein was added, the choline head group motion being considerably more sensitive only to the phosphatidylglycerol molecules. An analysis of the results showed that in all systems studied with protein concentrations of less than 33 wt %, fast exchange of all the lipids takes place between the bulk, protein-free bilayer phase and the protein-associating phase, with no evidence for lateral phase separation of lipid–protein domains with a lifetime of longer than approximately 1 ms. The number of acidic lipids interacting with the basic protein in these complexes is estimated to be between 15 and 28 molecules.

Investigations of lipid–protein interactions in reconstituted model membranes by NMR[1] or ESR spin-label methods have concentrated predominantly on the characterization of the motion of lipid fatty acyl chains with the hydrophobic surface of integral membrane proteins (Marsh & Watts, 1982; Rice et al., 1979; Oldfield et al., 1978; Seelig et al., 1981). Both magnetic resonance approaches are restricted to giving localized molecular information about the environment close to the site of probe attachment, and ^2H NMR spectroscopy is in principle the more versatile approach in that deuterium can substitute for protons at many specific positions in a phospholipid to give information about almost any part of the molecule. Although both methods have their own virtues and strengths in their applicability and motional time scales (Watts, 1981), ^2H NMR of specifically deuterated lipids is particularly suited to studying the anisotropy and rate of phospholipid head group motion at membrane surfaces in view of the minimal steric perturbation of deuterium substitution and thus close resemblence of the labeled and parent lipid. Therefore ^2H NMR has successfully been utilized in the study of the structure of membrane surfaces in pure lipid bilayers (Gally et al., 1975; Wohlgemuth et al., 1980; Seelig & Gally, 1976; Browning & Seelig, 1980), in phosphatidylcholine–cholesterol (Brown & Seelig, 1978), and in phosphatidylcholine–anesthetic mixtures (Boulanger et al., 1981). Also, we have shown that the ^2H NMR spectra of phospholipids specifically deuterated in their polar head groups are very sensitive to the interactions at the bilayer surface between different phospholipid types (Sixl & Watts, 1982, 1983).

Specific interactions at the membrane surface between proteins and different lipid types, distinguished by their head-group moiety, may be mediated either by steric means, by electrostatic means, or by both mechanisms. However, the structure of bilayer surfaces may be of importance in determining the specificity of lipid binding to membrane proteins (Watts et al., 1979; Knowles et al., 1981; Marsh & Watts,

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[1] Abbreviations: NMR, nuclear magnetic resonance; ESR, electron spin resonance; DMPC, 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine; DMPG, 1,2-dimyristoyl-*sn*-glycero-3-phospho-*rac*-glycerol; CSA, chemical shift anisotropy; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; DSC, differential scanning calorimetry; $\Delta\nu_Q$, deuterium quadrupole splitting.

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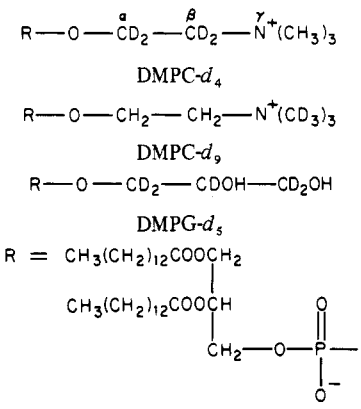
1982) or the activity of membrane-bound enzymes (Sanderman, 1978). In view of this importance, attention is now being given to studies of membrane surface associations between proteins and lipids, and recently the only systematic NMR study on the interaction between an integral membrane protein, cytochrome oxidase, and the head group of a membrane phospholipid has been reported (Tamm & Seelig, 1983). In this present work, we have studied the head group structure and motions of two naturally occurring lipid types, phosphatidylcholine and phosphatidylglycerol, in bilayers containing one of the major proteins from bovine myelin, basic protein. The phospholipids have been specifically deuterated at various positions in their polar head groups to enable us to use deuterium and phosphorus-31 NMR to study the rates and amplitude of motion using well-established methods (Seelig, 1977; Seelig & Seelig, 1980).

Myelin basic protein comprises about 30 wt % of the total myelin protein, and although it is a peripherally bound protein, there is some evidence that it partially penetrates the membrane bilayer and associates preferentially with acidic phospholipids (Boggs & Moscarello, 1978a; Boggs et al., 1982a). We have studied the protein-induced perturbations of the head-group motions in bilayers of the zwitterionic lipid, 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC), and the negatively charged 1,2-dimyristoyl-*sn*-glycero-3-phospho-*rac*-glycerol (DMPG), each separately and in equimolar mixtures. The protein effects were investigated and compared with the mutual motional distortion of one lipid head group by the other. We conclude that lipid–lipid interactions, as detected by the NMR measurements, are as significant in mixed lipid bilayers as the lipid interactions with basic protein. In addition, all the bilayer lipids are in fast exchange with each other on the NMR time scale, regardless of whether they are at the protein interface or are in the bulk lipid phase. A limit for the lifetime of basic protein complexes with acidic lipids, as suggested by Boggs et al. (1982a), was also estimated. It is further shown that deuterium NMR is a very suitable technique for detecting long-lived lateral phase separation of lipids in binary lipid mixtures.

Materials and Methods

Lipid Synthesis. The two lipids 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine and 1,2-dimyristoyl-*sn*-glycero-3-phospho-*rac*-glycerol were specifically deuterated in their head groups.



The syntheses of DMPC-d_4 and DMPC-d_9 were as previously described (Sixl & Watts, 1982). The sodium salt of DMPG-d_5 was prepared by the method of Harlos & Eibl (1980) using perdeuterated *rac*-isopropylideneglycerol in the esterification step. The latter compound was synthesized by dissolving perdeuterated glycerol in acetone and boiling under reflux for 6 h in the presence of catalytic amounts of *p*-toluenesulfonic acid, perdeuterated glycerol being produced by three exchanges of protonated glycerol against D_2O with Raney nickel as a catalyst.

Lipids were purified by silica acid chromatography as previously described (Watts et al., 1978) and appeared as single spots on thin-layer chromatograms. The lipid optical rotations $[\alpha]^{21}_{546}$, measured on a Perkin-Elmer 141 polarimeter in $CHCl_3/CH_3OH$, 2/1 v/v, were +7.80° for DMPC-d_9 and DMPC-d_4 and +8.78° for DMPG-d_5. Main gel to liquid-crystalline phase transitions, measured on a Perkin-Elmer DSC-2B in buffer (see below) at a rate of 10 °C/min and to an accuracy of ±0.5 °C, were 24.5 °C for DMPC-d_4 and DMPC-d_9 and 24.0 °C for DMPG-d_5; these transition temperatures are all within 1 °C of those measured for the non-deuterated, ^1H lipids synthesized in the same way and given in the literature (Silvius, 1982).

Protein Isolation. Bovine spinal cord was obtained fresh from the slaughterhouse and frozen immediately in liquid N_2. Myelin basic protein was extracted and purified as described by Deibler et al. (1972). The protein was further purified by chromatography on a Sephadex G-75 superfine column (3 × 90 cm) equilibrated with 10 mM HCl. The protein (M_r 18 300) was pure as judged by polyacrylamide gel electrophoresis in sodium dodecyl sulfate (Laemmli, 1970). Protein concentrations were measured spectrophotometrically by using $E^{1\%}_{276.4} = 5.64$ (Liebes et al., 1975).

Sample Preparation. Protein-free lipid dispersions were prepared as described previously (Sixl & Watts, 1982). All samples for NMR measurements were prepared in buffer made with deuterium-depleted water, the buffer being 2 mM Hepes, pH 7.5, containing 1 mM EDTA and 0.1 M NaCl.

Basic protein was reconstituted to produce homogeneous protein–lipid complexes by mixing a preformed lipid dispersion with the desired amount of a protein solution at about 40 °C (Boggs et al., 1980) by using the deuterium-depleted buffer. The basic protein solution was always adjusted to pH 7.4 with 1 N NaOH before mixing with the lipid. Values quoted for the lipid–protein ratios refer to the amount of protein added to the lipid dispersion.

Measurements. Deuterium NMR spectra were recorded at 46.1 MHz on a Bruker WH 300 spectrometer by employing single 90° pulses of 29-μs duration. As a consequence of the rather low spectrometer radio frequency power, the measurement of deuterium spin–lattice relaxation times T_1 by the inversion recovery method was only used when the NMR spectra were sufficiently narrow and when a complete inversion of the magnetization could be obtained.

Phosphorus-31 NMR spectra were recorded at 121.5 MHz under broad-band proton decoupling at a power of 7–10 W. Temperatures were controlled by a nitrogen gas flow to an accuracy of ±2 °C.

Results

Protein-Free Lipid Bilayers. Deuterium and phosphorus-31 NMR spectra of deuterated DMPC (DMPC-d_4 and DMPC-d_9), DMPG (DMPG-d_3 in which only the α- and β-segments were labeled), and mixtures of the two have been described and discussed in detail previously (Sixl & Watts, 1982). We have extended this work here by using the perdeuterated head group DMPG, DMPG-d_5. Also, the DMPG-d_3 used in the previous work possessed the D configuration in the glycerol head group, whereas DMPG-d_5 is a racemic mixture of both D and L diastereomers, which gave rise to small but significant changes in the deuterium NMR spectra. The spectrum of DMPG-d_5 at 25 °C is shown in Figure 1 (upper spectrum)

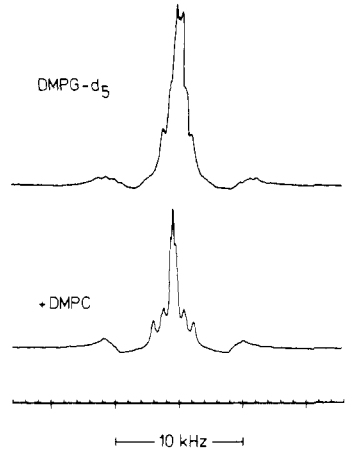


FIGURE 1: Deuterium NMR spectra (46.1 MHz) of DMPG-d_5 and of an equimolar mixture of DMPG-d_5 with DMPC. The amount of DMPG-d_5 is 20 mg; 50000 free induction decays were recorded with 29-μs (90°) pulses and a spectral width of 50 kHz. Temperature, 25 °C; pH 7.4; see the text for the buffer.

in which four quadrupole splittings of 12.3, 11.2, 10.1, and 9.2 kHz could be resolved for the α-CD_2 group. This is probably due to magnetic inequivalence of the deuterons and to the presence of two diastereomers. However, this result is in contrast to a previous report, in which only three distinct NMR signals were reported (Wohlgemuth et al., 1980). For convenience, a single average quadrupole splitting $\Delta\nu_Q$ for the α-methylene group of DMPG-d_5 will be given in the following ($\Delta\nu_Q$ = 10.6 kHz for DMPG-d_5 at 25 °C).

The γ-CD_2 group exhibited a complex behavior, in particular in mixtures with DMPC. In pure DMPG-d_5 bilayers, two quadrupole splittings of 1.2 and 0.6 kHz were observed at 25 °C. The difference between these two signals decreased at higher temperatures and only one spectral component with $\Delta\nu_Q$ = 0.6 kHz appeared at 50 °C. This is again in contrast to a previous study (Wohlgemuth et al., 1980), which reported both signals to become more separated with increasing temperature. Upon addition of an equimolar amount of DMPC to the DMPG-d_5 bilayers, a three-component spectrum for the γ-methylene group was observed (Figure 1) with quadrupole splittings at 25 °C of 1.9, 1.6, and 0.4 kHz. Increasing the temperature caused the two outer signals to collapse to a single line and at 50 °C two powder spectrum patterns remained with quadrupole splittings of 1.1 and 0.7 kHz.

DMPG-d_5–Basic Protein Membranes. Deuterium NMR spectra of DMPG-d_5 bilayers in the presence of basic protein are shown in Figure 2. Increasing the protein concentration to 50 wt % reduced the quadrupole splittings of all three deuterated head-group segments at 35 °C from 11.8 to 7.6 kHz (α-CD_2), from 2.9 to 2.0 kHz (β-CD), and from 0.8 to 0.4 kHz (γ-CD_2) as shown in Figure 3. These changes of $\Delta\nu_Q$ were only slightly temperature dependent for both pure lipid and protein-containing bilayers as illustrated in Figure 4. Figure 4 also contains information about the phase behavior of DMPG in the presence of basic protein. Well-resolved, single-component deuterium NMR spectra, characteristic of random dispersions of fluid lipid bilayers (Seelig, 1977), were obtained at temperatures of 25 °C and above regardless of whether basic protein was present or not. Also, all samples were in the gel phase below 23 °C, as demonstrated by the disappearance of the NMR signal for the α-methylene group and a collapse of the powder pattern from the β-CD and the γ-CD_2 segments into a featureless broad signal. No difference was found between cooling and heating scans. These results are similar to those from DSC and spin-label studies

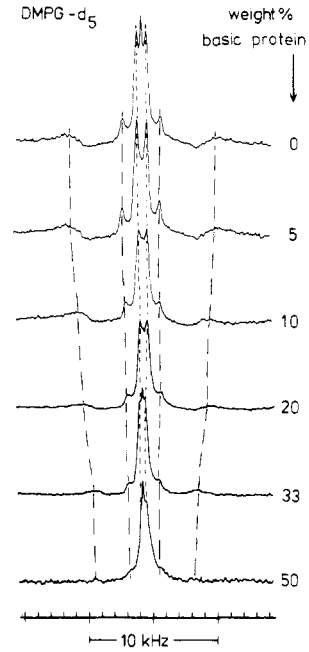


FIGURE 2: Deuterium NMR spectra (46.1 MHz) of DMPG-d_5 in the presence of different concentrations of basic protein. The amount of lipid was 15 mg and 20000 scans were recorded. Temperature, 35 °C; other conditions as for Figure 1.

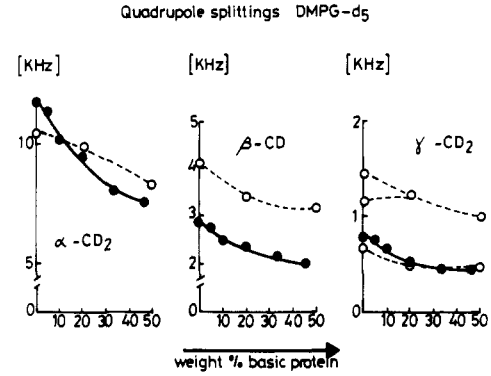


FIGURE 3: Quadrupole splittings of DMPG-d_5 for all three head-group positions as a function of basic protein concentration: filled symbols, DMPG-d_5; open symbols, DMPG-d_5/DMPC mixture (1/1 molar ratio); temperature, 35 °C.

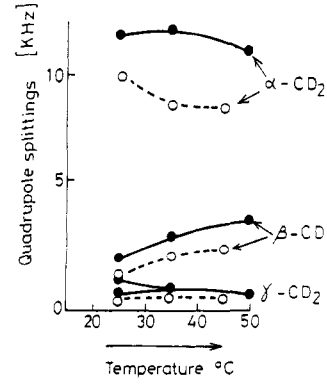


FIGURE 4: Temperature dependence of the quadrupole splittings for DMPG-d_5: filled symbols, DMPG-d_5; open symbols; DMPG-d_5 in the presence of 33% basic protein.

of DMPG–basic protein complexes (Boggs et al., 1982b).

Addition of basic protein to DMPG-d_5 membranes also affected the phosphorus-31 NMR spectra (Figure 5). At 35 °C, a decrease of the CSA value from −39 ppm for pure DMPG-d_5 to −25 ppm for a preparation with 50% protein was

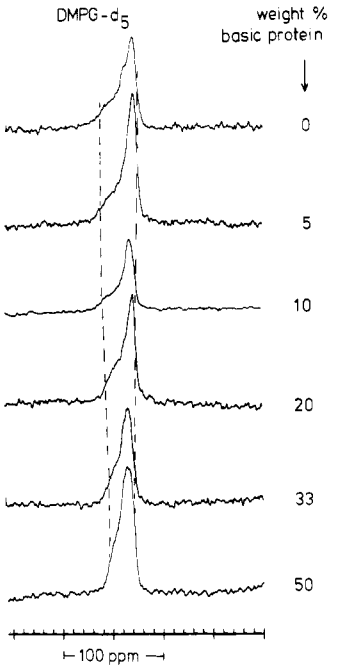


FIGURE 5: Proton decoupled phosphorus-31 NMR spectra (121.5 MHz) for DMPG-d_5 bilayers in the presence of increasing concentrations of basic protein. The spectra were recorded from the same samples used in Figure 2. A total of 1000 free induction decays were recorded for each spectrum. Temperature, 35 °C.

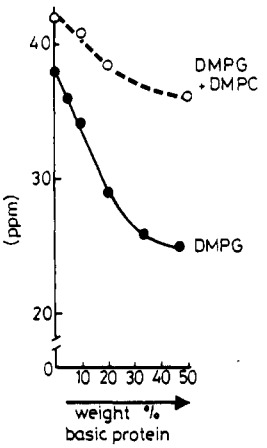


FIGURE 6: Dependence of the phosphorus-31 chemical shift anisotropy for pure DMPG-d_5 and its equimolar mixture with DMPC as a function of basic protein concentration at 35 °C.

measured (Figure 6). For each protein concentration, the spectral line shape indicated the lipid molecules to be in a bilayer arrangement (Seelig, 1978). However, in samples that contained 33% and more basic protein a very small isotropic signal could be detected, which was also seen in the deuterium NMR spectra. The origin of this isotropic signal is not known but is not thought to be due to the formation of small pure lipid aggregates, because DMPG was characteristically precipitated by the basic protein. A small population of inverted micelles, similar to those found in some biomembranes (Cullis & de Kruijff, 1979), cannot be excluded. It is also possible that some basic protein is associated with this lipid that is isotropically tumbling free in solution and not bound to the major population of multilamellar liposomes.

DMPC–Basic Protein Membranes. When basic protein was added to DMPC-d_4 or DMPC-d_9 bilayers, neither the deuterium quadrupole splittings nor the phosphorus-31 CSA were altered, nor did the spectral line shapes differ from those of pure DMPC (spectra not shown but given in Sixl & Watts (1982)), indicating that no or only a very weak binding of the protein to the phosphatidylcholine membrane surface occurs as shown by others (Boggs et al., 1982a).

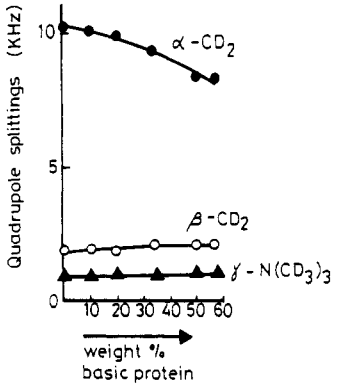


FIGURE 7: Quadrupole splittings of head group deuterated DMPC in a 1/1 mixture with DMPG as a function of increasing concentration of basic protein. Temperature, 35 °C.

Table I: Deuterium Spin-Lattice Relaxation Times (T_1) for DMPG-d_5 at 35 °C with and without Basic Protein, for Single Lipid Bilayers, and for Equimolar Mixed Bilayers with DMPC[a]

	T_1 (ms)	
	β-CD	γ-CD_2
DMPG-d_5	17 ± 1	19 ± 0
DMPG-d_5 + 20 wt % basic protein	12 ± 1	17 ± 0
DMPG-d_5/DMPC (1/1)	14 ± 1	21 ± 1
DMPG-d_5/DMPC (1/1) + 20 wt % basic protein	13 ± 2	19 ± 1

[a] Experimental details as for Figure 1.

Mixed DMPC/DMPG–Basic Protein Bilayers. The changes of deuterium quadrupole splittings for DMPG-d_5 in a 1:1 mixture with DMPC by addition of basic protein were similar to those in single-component DMPG-d_5 bilayers (Figure 3). At 35 °C, 50% protein decreased the $\Delta\nu_Q$ values from 10.5 to 8.3 kHz (α-CD_2) and from 4.1 to 3.2 kHz (β-CD). Of the three spectral components for the γ-methylene group with quadrupole splittings of 1.4, 1.2, and 0.7 kHz, two remained in the presence of basic protein with $\Delta\nu_Q$ values of 1.0 and 0.5 kHz.

Although composed of two different lipid types, DMPC/DMPG mixtures always exhibited single-component phosphorus-31 NMR spectra with CSA values of −42 ppm in the absence of protein and −36 ppm with 50% protein (Figure 6). We have shown previously that in aqueous dispersions of the two lipids, lateral phase separation does not occur (Sixl & Watts, 1982).

In equimolar mixtures of DMPC (either DMPC-d_4 or DMPC-d_9) with DMPG, basic protein had no effect on the quadrupole splitting of the β-methylene and the γ-$N(CD_3)_3$ group (Figure 7). A decrease of $\Delta\nu_Q$ was found for the α-CD_2 group; $\Delta\nu_Q$ was reduced from 10.2 kHz in protein-free lipid mixtures to 8.3 kHz in the presence of 56% basic protein. These changes are much less than those measured for the same lipid when mixed in an equimolar ratio with negatively charged DMPG (Sixl & Watts, 1982). In particular, the β-CH_2 segment, normally the most sensitive to surface effects, is virtually constant with protein content.

Deuterium Spin–Lattice Relaxation Times. Deuterium spin–lattice relaxation times T_1 were measured at 35 °C for the β-CD and the γ-CD_2 group of DMPG-d_5. The addition of basic protein had little effect of any of the relaxation times determined. Basic protein at 20 wt % decreased the T_1 values for the β-CD from 17 to 12 ms and from 19 to 17 ms for the

γ-CD_2 (Table I). The addition of unlabeled DMPC to DMPG had about the same effect on the β-CD group, for which T_1 was found to be 14 ms in a 1:1 mixture. T_1 for the γ-segment was slightly higher in the lipid mixture with a value of 21 ms. The presence of basic protein did not affect the relaxation behavior of the deuterated glycerol head group in mixtures with DMPC.

Analysis of Results. Information about the exchange rate of lipids between the bulk lipid phase and the complexes with basic protein can be derived from an analysis of the results of Figures 3 and 6. For fast, two-site exchange of lipid between the protein-free lipid part of the bilayer and the protein-associated phase, assuming that all the deuterated lipid contributes to the observed deuterium NMR spectra, then $n_t = n_c + n_f$, where n_t, n_c, and n_f are the number of total, complexed, and free lipid molecules per mole of protein in a reconstituted sample, respectively. The observed quadrupole splitting Q_0 will then be a weighted average of the quadrupole splittings in each of the two environments such that $Q_0 = (n_c/n_t)Q_c + (n_f/n_t)Q_f$. Substituting for n_f gives

$$Q_0 = n_c(Q_c - Q_f)/n_t + Q_f \quad (1)$$

Therefore, if the assumptions about exchange are valid, plotting $1/n_t$ against Q_0 will give a linear variation with an intercept at $1/n_t = 0$ of Q_f and a slope of $n_c(Q_c - Q_f)$. This treatment of the deuterium quadrupole splittings given in Figure 3 is shown in Figure 8a for each of the deuterons in DMPG-d_5 in single lipid bilayers containing increasing amounts of basic protein. A similar treatment can be performed for the phosphorus-31 CSA results of Figure 6 as shown in Figure 8b; here P is the phosphorus-31 CSA and the subscripts are the same as for eq 1, giving an identical form of eq 1 with Q replaced by P.

The linearity of the data confirms that the assumptions about fast lipid exchange between lipid–protein complexes and the bulk lipid are valid. Also, at $1/n_t = 0$, the extrapolated value of Q_f (P_f) is close to the experimentally determined value of Q_0 (P_0) for pure lipid bilayers; these values are also plotted in Figure 8. Some deviation from linearity is observed at the highest protein concentration measured ($1/n_t = 0.033$), and this may be due to saturation of lipid binding sites.

A similar analysis has been attempted with the results obtained from DMPG/DMPC mixed lipid bilayers on binding of basic protein. The values of Q_0 and P_0 do vary linearly with $1/n_t$, implying fast exchange of all the lipids in these complexes. However, a number of assumptions must be made about the way in which each lipid type interacts with the protein, in particular that the phosphatidylcholine has essentially no interaction with the protein, as may be the case from the results shown in Figure 7. A further complication is that the composition of the mixed bilayer will change from an equimolar lipid mixture to a more rich DMPC bilayer due to basic protein interacting with only the acidic lipids. This kind of lateral phase separation is not long-lived on the NMR time scale since only single component spectra are observed. Therefore some account must be made for the changes in the DMPC deuterons as there becomes less DMPG with which to interact. However, we feel that the analysis is complex and we require additional, possibly independent, information before the data can be analyzed more precisely.

It is clear that in the mixed lipid complexes, DMPC (DMPC-d_4 and DMPC-d_9) does sense the DMPG molecules from the results of Figure 7. Protein-free *and* protein-containing bilayers of labeled DMPG/DMPG both show very similar spectral parameters to those reported by us previously (Sixl & Watts, 1982), and these quadrupole splittings are very

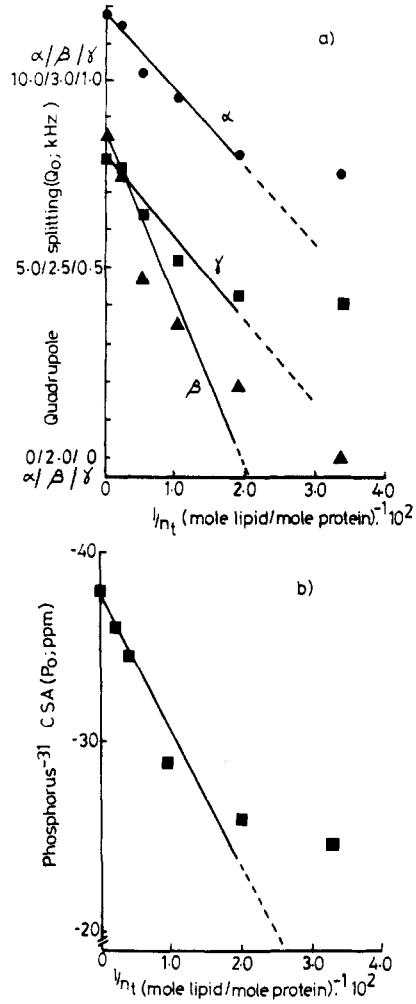


FIGURE 8: Variation of the observed spectral parameters [quadrupole splittings, Q_0 in (a); phosphorus-31 CSA, P_0 in (b)] from the data in Figure 3 (to give plot a) and Figure 6 (to give plot b) for the head group of DMPG-d_5 as a function of the reciprocal of the lipid to basic protein mole ratio ($1/n_t$) according to eq 1. The points at $1/n_t = 0$ are Q_f and are experimentally determined values from protein-free bilayers.

different from those for pure DMPC bilayers. In addition, in mixed DMPC/DMPG-d_5 bilayers, the acidic lipid detects the presence of the basic protein (Figure 3), but the DMPC molecules are again much less affected (Figure 7). Therefore the existence of any DMPG–protein complexes in different bilayers remote from pure DMPC bilayers is not possible, especially since single-component NMR spectra are detected in all the systems investigated.

Discussion

A very detailed model has been proposed for the interaction of myelin basic protein with phospholipid bilayers (Boggs et al., 1982a). From ESR spin-label and differential scanning calorimetry (DSC) studies it was concluded that basic protein preferentially binds to the acidic components in mixtures of negatively charged and zwitterionic lipids (Boggs et al., 1977a) and partly penetrates into the hydrophobic membrane interior (Boggs & Moscarello, 1978a,b). The high affinity of basic protein for acidic phospholipids (Demel et al., 1973; Gould & London, 1972) and the low degree of binding to phosphatidylcholine (Boggs & Moscarello, 1978a) suggest that its interaction with bilayer membranes is primarily electrostatic in nature. The electrostatic binding of a macromolecule to a membrane surface might be anticipated to induce rather large perturbations of the structural organization and motional

rate of the phospholipid head groups, which is where the negative charge and the possible binding site are located.

Fast exchange of DMPG lipids with the interface of basic protein is suggested by the analysis of the NMR results and the fit of eq 1 where the measured parameters are linear with the protein to lipid mole ratio in Figure 8. Deviation from linearity of the data in Figure 8 occurs between values of n_t of 54:1 and 34:1 and may indicate saturation of the number of protein binding sites and that some protein is free in solution at high protein concentration. A complete solution of eq 1 is not possible and thus no numerical values of either n_c or Q_c (or P_c) can be deduced directly. However, the number of lipids, n_c, interacting with the protein has been estimated by using a variety of different methods. Basic protein has 31 positively charged amino acids (Lys, Arg) at physiological pH, and it has been shown that the molar ratio of negatively charged lipids to protein in a water-insoluble complex was 23:1 (Palmer & Dawson, 1969). Also, the number of positive charges on basic protein that may be available for acidic lipid associations has been determined from competitive Mn^{2+} binding using ESR methods to be 26 ± 5 (Boggs et al., 1981). From phase diagrams derived from DSC experiments it has been shown that 27–34 acidic lipids interact with basic protein (Boggs et al., 1977b). Finally, recent stopped-flow measurements of basic protein association with phosphatidylglycerol vesicles have shown that 24 lipids interact with each protein monomer (Lampe et al., 1983). Although these various experiments may be measuring protein–lipid associations of dissimilar physical origin, there seems to be a concensus for the number of lipid molecules found to be interacting with the protein. Using an average value from all these experiments for n_c of 27 molecules and assuming it is the same for each part of the lipid, one can calculate a value of Q_c (and P_c) from the slopes of Figure 8. The quadrupole splittings for the lipid segments when at the protein interface were calculated to be 3.5, 1.25, and 0.04 kHz for the α, β, and γ head-group segments of DMPG, respectively, and P_c, the CSA for lipid at the protein interface, has a value of +3.5 ppm. These results imply that all parts of the lipid head groups may be much more disordered or take up different average conformations when at the protein interface than when in a protein-free environment. Alternatively, fast local motion (on the NMR time scale) of the CD bonds and the phosphate group about a larger angular range of diffusional tensors on the protein surface than in protein-free bilayers would also decrease the measured spectral anisotropies. Fast exchange of lipids throughout the complexes then averages the much smaller protein-induced anisotropies with the much larger characteristic bilayer powder patterns, decreasing the observed spectral anisotropy. A reduction in the spectral anisotropy for all the head-group segments of an unsaturated phosphatidylcholine on reconstitution with cytochrome *c* oxidase has been observed in deuterium, nitrogen-14, and phosphorus-31 NMR experiments, although in this work no lipid–protein titrations were systematically performed (Tamm & Seelig, 1983).

Since all the values of Q_c and of P_c derived from eq 1 as discussed above are rather close to zero, it is possible that the lipids at the protein interface are completely disordered (but still have fast motion) and that there is a common value of zero of all the CD quadrupole splittings and the phosphorus CSA. If this is the case, values for n_c of 18.2, 15.1, and 28.5 molecules can be derived from the slopes of Figure 8 for the α-, β-, and γ-segments of DMPG and 24.5 molecules from the phosphorus CSA data. The average value calculated from these three independent estimates of n_c is 22 ± 3 (SEM) molecules. It is likely that each head-group segment, as well as the phosphorus-31 atom, interacts with basic protein to the same extent, thereby giving the same value of n_c for each set of data. A solution for eq 1 to fit all sets of data and making this assumption is $Q_c \simeq P_c \simeq 0$.

The above interpretations do not allow for perturbation of lipids remote from the site of immediate contact with the protein, so-called "second shell" effects with integral proteins. If the value of $1/n_t$ at which a measured parameter in Figure 8 deviates from linearity indicates saturation of the protein binding sites, then all the lipid is complexed to the protein at this point. From the intercepts for the four sets of data, and the slopes of the linear parts of Figure 8, values for n_c of between 49 and 64 molecules are obtained from eq 1. This interpretation of the data leads to $Q_c \neq 0$, with approximate values for Q_c of 8, 2, and 0.4 kHz for the α-, β-, and γ-deuterated segments and −24 ppm for the phosphorus CSA. In complexes containing excess protein therefore, the bound and free lipids may be in slow exchange with each other on the ^2H NMR time scale.

Fast exchange of all lipids (on the NMR time scale) in mixed lipid bilayers either with each other or with the protein is confirmed by the single component nature of all the deuterium and phosphorus-31 spectra at higher lipid–protein ratios. The anisotropy difference, given by $Q_f - Q_c$, gives a lower limit for the exchange of deuterons between the protein surface and the bulk phase. The widest range of values of Q_f for DMPG is between 11.8 kHz (for the α-CD_2) and 0.79 kHz (for the β-CD) at 35 °C from Figure 3. Assuming a value for Q_c of close to zero (between 3.5 and 0.04 kHz) implies that lipid exchange is faster than 10^4–10^5 s^{-1}. The lipid hopping frequency in pure lipid bilayers is about 3 orders of magnitude faster than this limit, with a value of 10^7–10^8 s^{-1} (Galla et al., 1979). In agreement with deuterium studies of integral protein–lipid interactions (Seelig et al., 1982), the present results suggest that in addition to binding to basic protein, the negatively charged lipids are in fast exchange (on the time scale of the ^2H NMR method) with the other lipids. The ability of lipids to bind protein while maintaining relatively unrestricted lateral mobility is likely to be a factor contributing to the dynamic stability of membranes.

Limited restriction of the segmental motional *rate* of the DMPG-d_5 head group on interacting with basic protein is evident from the measurements of deuterium spin–lattice relaxation times T_1. From the results in Table I, rotational correlation times can be calculated by assuming small order parameters (less than 0.1) of the CD bond (Brown et al., 1979) and were found to be increased from 1.1×10^{-10} to 1.3×10^{-10} s for the γ-methylene segment and from 1.3×10^{-10} to 1.9×10^{-10} s for the β-CD group on the addition of 20 wt % protein to pure DMPG-d_5 bilayers. Due to instrumental limitations, reliable T_1 values could not be measured for the α-methylene segment of DMPG-d_5, but they appeared to decrease in protein-containing bilayers. There is clearly a problem of assigning a suitable value to the deuterium order parameter for each segment in such calculations of correlation times. However, it seems reasonable to use this approach to estimate the *rate* of head-group motion, which is almost isotropic when using a procedure that, for small values of S_{CD}, is relatively insensitive to the *amplitude* of segmental motion. No detectable changes were seen in the T_1 measurements for any DMPC head-group segments on adding protein to DMPG/DMPC bilayers, again demonstrating the selectivity of the interaction. Our findings therefore confirm previous carbon-13 NMR studies in which the NMR line-width changes for the

PG head group in vesicles were interpreted in terms of a decrease in the rate of glycerol head-group motion on binding of basic protein (Stollery et al., 1980). In that work vesicle aggregation by basic protein, which has recently been demonstrated by light scattering experiments (Young et al., 1982), would induce the same observed NMR line-width changes.

The deuterium NMR spectra for the γ-CD_2 segment of pure DMPG-d_5 bilayers are not understood in detail. The two components, which are observed for pure DMPG-d_5 bilayers at temperatures near the main phase transition, are motionally averaged at 35 °C, giving rise to a single powder pattern with a quadrupole splitting of 0.8 kHz. Magnetic inequivalence of the two deuterons or the presence of two slowly exchanging head-group conformations that become quickly exchanging at high temperatures (Büldt & Wohlgemuth, 1981) could account for this behavior. Similarly, the observation of three quadrupole splittings for the γ-CD_2 of DMPG-d_5, when mixed with an equal amount of DMPC (Figure 1), is even less clear. A complicating factor is the presence of two diastereomers in the glycerol head group. Experiments with 1,2-dimyristoyl-*sn*-glycero-3-phospho-*sn*-glycerol are presently being carried out to resolve possible effects resulting from different behavior of the diastereomers.

The quadrupole splittings for *all* the head group deuterated segments of DMPC, especially the β-CD_2 and γ-$N(CD_3)_3$ segments, have been shown to be extremely sensitive to the composition of the lipid matrix (Sixl & Watts, 1982, 1983). However, the addition of basic protein to DMPC/DMPG bilayers caused only a small effect on the α-CD_2 segment of the choline head group (Figure 7) and may reflect dilution of the γ-$N(CD_3)_3$ motional perturbation by DMPG since the decrease in quadrupole splitting at 60 wt % basic protein approaches a value similar to that for pure DMPC bilayers (Sixl & Watts, 1982). The β-CD_2 and the γ-$N(CD_3)_3$ groups showed no sensitivity to basic protein. All the spectra recorded were single component, demonstrating that no long-lived lateral separation of lipid occurred in any complex. Since the time needed to average the spectral anisotropy of laterally phase separated lipids is 1 ms, no lipids exist in any single environment for longer than this time period. Lateral phase separation of lipids in bilayers has been demonstrated by phosphorus NMR studies of mixed bilayers (Arnold et al., 1981). In addition, the protein does not extract the negatively charged lipids from the mixed bilayers, since the DMPC molecules still monitor their interactions with the DMPG head groups in the presence of protein; such lipoprotein assemblies might be expected to be small and therefore yield isotropic NMR spectra that are not observed. A very limited degree of basic protein binding to phosphatidylcholine dispersions at the same pH as in our experimnets has been demonstrated by light scattering (Smith, 1977). From the lack of choline head-group perturbation we observe for DMPC, it is concluded that such associations are not electrostatic, since light scattering experiments are most sensitive to vesicle size and the refractive index of the hydrophobic bilayer.

From model studies it has been suggested that preferential binding of acidic lipids to basic protein to form protein–lipid complexes may be responsible for the asymmetric structure of myelin membranes (Boggs et al., 1982). Our results clearly reinforce the idea that complexes are formed in mixed lipid bilayers and suggest that at physiologically relevant lipid to protein ratios these complexes have a lifetime of shorter than 1 ms. However, our mixed lipid experiments show that basic protein does not confer either an absolute or a partial asymmetry on the DMPC/DMPG model membranes we have employed, since the bilayers are homogeneous and the major perturbation sensed by one lipid is from the presence of the other type in the same bilayer. The dynamic specificity of acidic lipid association with myelin basic protein may therefore be essential in stabilizing the molecular arrangement and integrity of the myelin sheath.

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Partition of Fatty Acids and Fluorescent Fatty Acids into Membranes†

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ABSTRACT: We have measured the membrane/water partition of free fatty acids, of a fluorescent analogue, and of chlorpromazine into plasma membranes from lymphoma, platelets, red cells, and liposomes of egg phosphatidylcholine. Three different methods were used: hygroscopic desorption [Conrad, M. J., & Singer, S. J. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 5202–5206; Conrad, M. J., & Singer, S. J. (1981) *Biochemistry 20*, 808–818], centrifugation, and fluorescence titration. The partition coefficients for chlorpromazine and all fatty acid/membrane combinations were in the range 10^2–10^5. No significant partition difference was observed between liposomes and plasma membranes. These results suggest, therefore, that fatty acids and chlorpromazine can readily partition into artificial and cell plasma membranes.

Much of our understanding of membrane structure and function has been inferred from the interaction of amphipathic molecules with the lipid bilayer of the membrane (Marsh, 1981; Yguerabide & Foster, 1981). In most of these studies, it is assumed that the hydrophobic portion of the molecule or probe monitors the internal structure of the membrane. It follows that the hydrophobic portion of the amphipath partitions into the bilayer so that it is shielded from the aqueous environment. With the exception of artificial liposomes (Lesslauer et al., 1972; Podo & Blaisie, 1977), however, direct proof (for example by X-ray or NMR methods) of the internal location of the probes is lacking.

Recently, Conrad & Singer (1979, 1981) have called into question one of the basic tenets of membrane probe studies. These authors raised the intriguing possibility that although amphipaths can partition into artificial liposomes, they are excluded from the internal regions of biological membranes. Instead, they suggested the generally observed associations of probes with membranes result from the interaction of amphipathic micelles with the surface. It was hypothesized that virtually all indirect observations of membrane/probe associations could be interpreted in terms of these micelles.

Conrad & Singer (1981) argued that centrifugation, filtration, or spectroscopic probe signal enhancement methods are inadequate to determine the membrane/water partition of amphipaths, since in attempting to separate membrane-associated from free amphipath no provision is made to eliminate the formation of membrane-bound micelles. To overcome this problem, they developed a technique called hygroscopic desorption in which the aqueous phase is removed from the membranes and replaced by air. Using this technique, it was found that four amphipaths, chlorpromazine, methyl chlorpromazine, 2,4-dinitrophenol, and 1-decanol, exhibited large partition coefficients into artificial liposomes. The partition into red cell and lymphoma plasma membranes, however, was at least 10^4 times lower and was, in fact, consistent with zero. With these observations in mind, Conrad & Singer (1981) suggested that no amphipath, including fatty

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