Fragmentation of Phospholipid Bilayers by Myelin Basic Protein†

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ABSTRACT: Human myelin basic protein (MBP) is shown to disrupt multilamellar phosphatidylcholine bilayers into small lipoprotein particles in a manner similar to the cytolytic peptide melittin (Dufourc, E. J., Smith, I. C. P., & Dufourcq, J. (1986) *Biochemistry 25,* 6448–6455). This bilayer fragmentation, as monitored by ^{31}P nuclear magnetic resonance, is temperature-dependent and completely inhibited by the presence of small amounts of negatively charged phosphatidylserine. The stabilizing property of phosphatidylserine is lost with the neutralization of its negative charges upon membrane binding of cationic species such as calcium ions. No MBP-induced fragmentation is observed with bilayers of negative or zwitterionic lipid mixtures which mimic the myelin lipid composition. The membrane fragmentation observed *in vitro* in the presence of MBP could play a role *in vivo* in demyelinating diseases.

Myelin basic protein (MBP)[1] is the predominant extrinsic protein in myelin, and the interaction between MBP and lipid bilayer membranes has been intensively studied using a wide variety of experimental techniques. This interest has been driven by a desire to understand better the role of MBP in the compaction and stabilization of the myelin sheaths found in the central nervous systems (CNS). On the medical side, MBP exhibits antigenic properties in the autoimmune response believed by many to occur in demyelinating diseases such as multiple sclerosis (MS), which lead ultimately to the destruction of the myelin structures [for a review see Martin et al. (1992)]. It is well established that MBP binds to lipid bilayers having negatively charged (acidic) phospholipids (Demel et al., 1973; Boggs & Moscarello, 1978; Sixl et al., 1984; Sankaram et al., 1989) and probably stabilizes opposing cytoplasmic surfaces of the myelin membrane (Boggs et al., 1981). It is also widely assumed that MBP does not bind to zwitterionic lipid bilayers containing only phosphatidylcholine (PC), although binding to phosphatidylethanolamine (PE) bilayers has been observed (Boggs & Moscarello, 1978).

This paper presents conclusive evidence that, contrary to general belief, MBP does interact with PC bilayers and profoundly affects their spatial organization. The binding and ^{31}P NMR studies described here show that when MBP interacts with bilayers composed of PC molecules, it causes them to fragment into MBP–PC aggregates, in a way strikingly similar to that of cytolytic melittin. This experimental result is a useful addendum to our knowledge of the systematics of interaction between extrinsic proteins and lipid bilayer membranes. It also leads us to propose a possible scenario for the onset of demyelinating diseases that should merit further research.

MATERIALS AND METHODS

Binding Experiments. Myelin was isolated from normal human white matter, and MBP was extracted and purified using previously described methods (Deibler et al., 1972). All lipids were purchased from Avanti Polar Lipids (Birmingham, AL), except cholesterol which was from Sigma (St. Louis, MO). Myelin-like membranes were prepared according to the lipid composition, expressed in weight percentage, of human brain myelin (Wood & Moscarello, 1984). Negative membranes contained POPC (10.6%), POPE as ethanolamine phosphatides (26.1%), POPS (4.5%), cerebrosides (21.5%), sulfatides (3.6%), sphingomyelin (7.5%), and cholesterol (26.2%), but no phosphatidylinositol. Zwitterionic membranes were identical, except that POPS and sulfatides were replaced by additional POPC (8.1%). Multilamellar liposomes were prepared from chloroform solutions of the various lipids mixed in an appropriate ratio (≈1.5 mg of total lipids) containing 0.0001 mol% phosphatidylcholine tritiated on one methyl group, 1,2-dipalmitoylphosphatidyl[N-*methyl*-^3H]choline (^3H-DPPC) from Amersham. The solvent was removed by evaporation under N_2. The solid residues were dried under high vacuum (10^{-2} mmHg) for 12 h and then dispersed with continuous vortexing at 40 °C in 200 μL of Tris buffer (40 mM, pH 7.0, with 10 mM NaCl) containing appropriate amounts of MBP, giving 10 mM lipid dispersions. The resulting samples were submitted to five freezing (liquid nitrogen) and thawing (40 °C) cycles and centrifugated at 20 °C (15000*g*, 1 h), and the optically clear supernatants were assayed for phospholipid and MBP contents. Lipid content was assessed by ^3H radioactivity measurements with ^3H-DPPC on 10-μL aliquots (5000–8000 cpm were obtained for 10 mM lipid prior to centrifugation). Indeed, this method relies on the assumption that the probe, ^3H-DPPC, is evenly distributed through the lipid phase(s). MBP was quantified by the Lowry method (Lowry et al., 1961), and the pellet-bound MBP was calculated by substracting the obtained values of free MBP from the total MBP added.

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[1] Abbreviations: CNS, central nervous system; MBP, myelin basic protein; MS, multiple sclerosis; NMR, nuclear magnetic resonance; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; DPPC, dipalmitoylphosphatidylcholine; POPC, 1-palmitoyl-2-oleoylphosphatidylcholine; POPE, 1-palmitoyl-2-oleoylphosphatidylethanolamine; POPS, 1-palmitoyl-2-oleoylphosphatidylserine; Tris, tris(hydroxymethyl)aminomethane.

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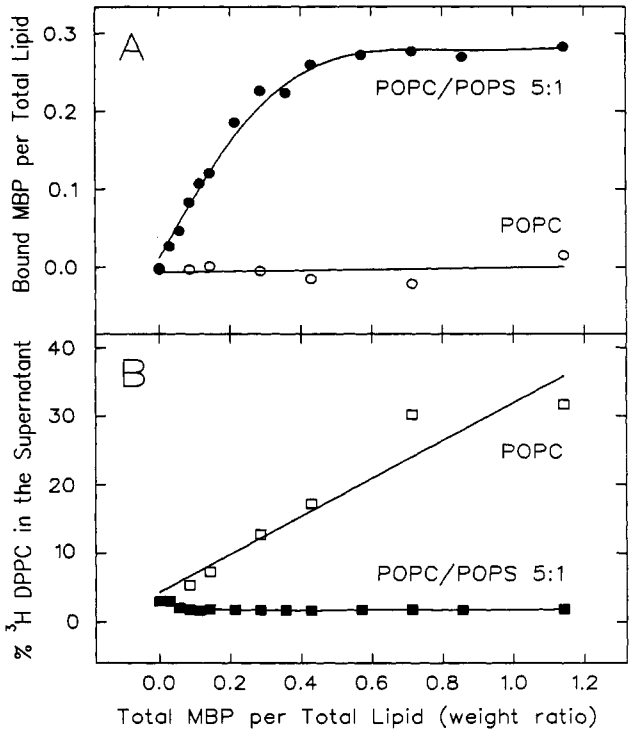

FIGURE 1: (A) Binding of human myelin basic protein to multilamellar dispersions of phosphatidylcholine (○) and phosphatidylcholine/phosphatidylserine 5:1 (mol/mol) (●). Total and bound MBP are expressed as protein-to-lipid weight ratio. (B) Solubilization of phosphatidylcholine (□) and phosphatidylcholine/phosphatidylserine 5:1 (mol/mol) (■) multilamellar dispersions by human myelin basic protein. The data are expressed as the percentage of tritiated DPPC (see Materials and Methods) remaining in the supernatant versus the amount of total MBP.

^{31}P NMR Experiments. Liposomes were prepared as described in the binding experiments, with a total lipid concentration of 40–70 mM. The lipid dispersions were transferred into the NMR tubes without centrifugation. ^{31}P NMR experiments were done at 81 MHz on a Bruker MSL-200 spectrometer and 121.5 MHz on a Bruker MSL-300. Spectra were acquired with a dwell time of 20 μs with 2048 (81 MHz) or 4096 (121.5 MHz) data points and a repetition time of 3 s. A Hahn echo pulse sequence (Rance & Byrd, 1983) with Waltz decoupling (during acquisition) was employed, with a pulse separation τ of 60 μs. We used a 10-mm diameter solenoid coil with pulse length of 4 μs at 81 MHz and 5-mm diameter tubes with pulse length of 3 μs at 121.5 MHz. One to six hours was allowed for the equilibration of the temperature, and spectra were acquired over periods of 2–8 h. The free induction signal was shifted by some fraction of the dwell time to ensure that the effective starting time for the Fourier transform corresponded to the top of the echo.

RESULTS AND DISCUSSION

MBP Binding to POPC and POPC/POPS Membranes. We have performed centrifugation experiments on MBP solutions containing various amounts of multilamellar liposomes of POPC, either pure or mixed with 16.7% acidic POPS. As expected from previous studies (Boggs & Moscarello, 1978; Ter Beest & Hoekstra, 1993), MBP binds to negative POPC/POPS membranes. The amount of MBP bound to the pelleted negative multilamellar lipid bilayers, depends on the protein to lipid ratio. As shown by the binding curve of Figure 1A, a saturation behavior occurs around a protein to lipid weight ratio of about 0.3 (1.2 mol % MBP), giving about 1 18.4-kDa MBP for 14 POPS molecules. The corresponding curve obtained with pure POPC membranes confirms that MBP does not bind to the *pelleted* POPC bilayers (Figure 1A). Yet POPC does interact with MBP, as shown by the appearance in the optically clear supernatant of nonpelleted lipids, as probed by radioactivity measurements with tritiated phosphatidylcholine (Figure 1B). Supernatant lipid is found to increase linearly with the addition of MBP up to 30% of the total lipids for a 1:1 weight ratio of protein per lipid. Linear regression analysis yields a value of ≈0.3 mg of lipid/mg of MBP, giving about 7 lipid molecules in the supernatant per unit of MBP. Thus, it appears that MBP is able to fragment large multilamellar liposomes of phosphatidylcholine into smaller "solvated" lipid/protein complexes. Such solubilization by MBP is not observed with PS-containing membranes (Figure 1B).

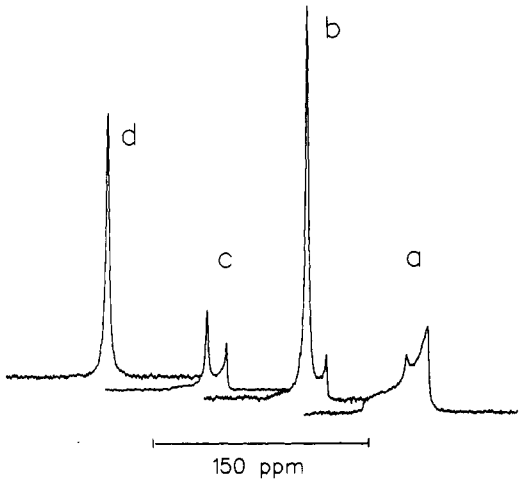

FIGURE 2: ^{31}P NMR spectra at 121.5 MHz of pure POPC dispersions in the absence (a) and the presence (b) of myelin basic protein (1 mg of protein/mg of total lipid) at 37 °C. After centrifugation at 37 °C of the MBP-containing sample (5000*g*, 15 min), ^{31}P NMR spectra were recorded under the same conditions for the pellet (c) and the supernatant (d). Spectra a and b are normalized according to their areas.

MBP Interaction with POPC and POPC/POPS Membranes As Seen by ^{31}P NMR. The lipid/protein complexes indicated by the centrifugation experiments can also be observed using ^{31}P NMR, which is a sensitive probe of phospholipid organization (Cullis & De Kruijff, 1979). The ^{31}P NMR spectrum of pure PC aqueous dispersions at 37 °C covers a frequency range of about 50 ppm and is typical of large particles (≈1 μm) of phospholipid bilayers in the fluid state (Figure 2, spectrum a). Under similar conditions, smaller particles (radius <2000 Å) of aggregated bilayers are associated with NMR spectra showing a reduced overall width. Ultimately, very small objects such as those detected in the above binding study (micelles of small vesicles) should give a narrow isotropic resonance centered at the lipid phosphorus chemical shift position and superimposed on a residual powder pattern (Burnell et al., 1980). The spectrum obtained with MBP-containing POPC membranes (Figure 2, spectrum b) shows that this is indeed the case. However, isotropic resonances can also arise from *large* aggregated lipidic particles containing highly curved bilayer structures (Batenburg et al., 1987). In the present study, NMR control experiments carried out after centrifugation of the samples indicate that this hypothesis can be ruled out, since the isotropic phase remains in the optically clear supernatant (spectrum d). Indeed, the pellet produces a bilayer spectrum, with a remaining isotropic component due to residual supernatant (spectrum c). Thus,

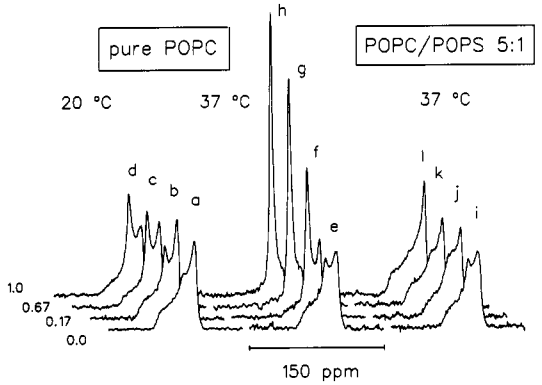

FIGURE 3: Effect of human myelin basic protein on the microstructure of model membranes as monitored by ^{31}P NMR at 81 MHz. Pure POPC dispersions at 20 °C (a–d) and 37 °C (e–h), and POPC/POPS 5:1 (mol/mol) dispersions at 37 °C (i–l) prepared in the absence of MBP (a, e, i) and in the presence of 0.17 (b, f, j), 0.67 (c, g, k) and 1 (d, h, l) mg of MBP/mg of total lipid (1:1 weight ratio ≈24 lipid molecules/MBP). The NMR spectra are normalized according to their areas. Note the higher resolution of the data recorded at 121.5 MHz (Figure 2), i.e., the separation between the isotropic resonance and the edge of the powder pattern.

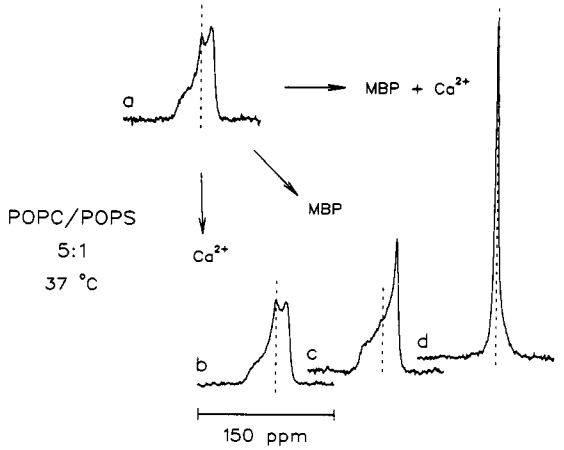

FIGURE 4: Influence of calcium on MBP binding to model membranes as monitored by ^{31}P NMR at 81 MHz. POPC/POPS 5:1 (mol/mol) dispersions at 37 °C, prepared in the absence (a) or in the presence of (b) 50 mM $CaCl_2$, (c) MBP, or (d) MBP and 50 mM $CaCl_2$, with a protein to lipid weight ratio of 1:1. The NMR spectra are normalized according to their areas. The chemical shift of the isotropic resonance is represented by the dotted lines.

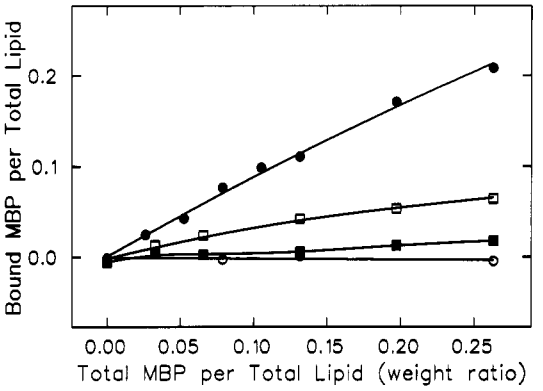

FIGURE 5: Binding of human myelin basic protein to myelin-like membranes. The negative myelin-like membranes (□) contained phosphatidylcholine (10.6%), phosphatidylethanolamine (26.1%), phosphatidylserine (4.5%), cholesterol (26.2%), sphingomyelin (7.5%), cerebrosides (21.5%), and sulfatides (3.6%). The lipid composition of the zwitterionic myelin-like membranes (■) was that of the negative membranes in which phosphatidylserine and sulfatides were substituted by additional phosphatidylcholine. Data obtained with multilamellar dispersions of phosphatidylcholine (○) and phosphatidylcholine/phosphatidylserine 5:1 (mol/mol) (●) are also included. Total and bound MBP are expressed as protein-to-lipid weight ratio.

large multilamellar lipid bilayers and small particles of MBP-bound POPC, can be monitored *in situ* by their broad and narrow NMR spectral components, whose relative intensities are proportional to the amounts of the two kinds of hydrated lipid. In good agreement with the binding studies, the data of Figure 3 show clearly that this membrane fragmentation is MBP-dependent. In addition, this effect appears to be temperature-dependent and is more pronounced at physiological temperatures (37 °C) than at the temperature used in the binding experiments (20 °C). For a 1:1 protein to lipid weight ratio at 37 °C, we find that up to 70% of the total lipids are in the solubilized aggregates (Figure 3, spectrum h). Despite previous evidence of strong MBP binding to the negatively charged POPC/POPS bilayers, protein-induced disruption of these membranes is still not observed at 37 °C. The presence of MBP in the lipid suspension actually counteracts a weak—but significant—spontaneous "vesiculation" (<5% of total lipids) observed in the absence of protein (Figure 3, spectra e and i). This phenomenon clearly disappears upon MBP binding to the negative lipid phase (Figure 3, spectra j–l). A similar MBP-induced "stabilization" of small lipid vesicles was previously described in a study with mixtures of brain phospholipids (Fraser et al., 1989).

MBP Interaction with POPC/POPS Membranes in the Presence of Calcium. The binding and NMR data show that MBP is able to interact with either zwitterionic (POPC) or negatively charged (POPC/POPS) membranes but that complexes of MBP with multilamellar bilayers of pure POPC are unstable and break down into small lipid/protein particles. In such cases, an electrostatic stabilization of the MBP/bilayer complex by the negative surface charge of the PS-containing membranes is strongly suggested. To investigate this hypothesis further, these bilayers were neutralized with calcium ions which have been shown to bind to POPC/POPS membranes (Roux & Bloom, 1990), thus competing with MBP for the negative membrane-binding sites. As shown in Figure 4, the addition of MBP to PS-containing bilayers in the presence of Ca^{2+} at a concentration where the PS charges are neutralized (Roux & Bloom, 1990) results in a large isotropic peak in the ^{31}P NMR spectrum (spectrum d), not observed with calcium ions alone (spectrum b). Hence, the MBP-induced fragmentation of multilamellar liposomes can be observed with POPC/POPS mixtures provided the negative membrane surface charge is neutralized upon binding of cationic molecules such as Ca^{2+} ions. These results support the currently accepted idea that ionic interactions with negative lipids are essential to the formation of the highly stable MBP/bilayer complexes found in myelin (Boggs et al., 1982).

MBP Interaction with Myelin-like Membranes. MBP binding studies were repeated with zwitterionic and negative model membranes mimicking the myelin lipid composition. For a MBP to lipid weight ratio of 0.2, we find that about 27% of the protein binds to negative myelin-like membranes, versus 80% for POPC/POPS 5:1 membranes (Figure 5). This difference is probably due to the fact that our negative myelin-like membranes contain only 6 mol % negatively charged lipids versus 16.7% for the POPC/POPS liposomes. Zwitterionic myelin-like membranes contain no negatively charged lipids and bind much less MBP. For the same protein to lipid weight ratio, bound MBP is less than 10% for these membranes. For both types of myelin-like membranes, the amount of lipids remaining in the supernatant, as probed with tritiated PC,

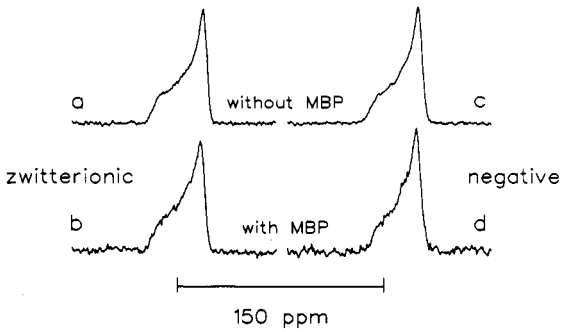

FIGURE 6: Effect of human myelin basic protein on zwitterionic (a, b) and negative (c, d) myelin-like membranes at 37 °C as monitored by ^{31}P NMR at 81 MHz. Membranes were prepared in the absence (a, c) or in the presence (b, d) of 1 mg of MBP/mg of total lipid. The NMR spectra are normalized according to their areas.

is always less than 5% at all MBP concentrations, indicating that there is no MBP-induced fragmentation of the myelin-like membranes. In good agreement, ^{31}P NMR spectra of negative and zwitterionic myelin-like membranes indicative of fluid lipid bilayers[2] at 37 °C do not contain any isotropic components in either the absence or the presence of MBP (Figure 6). Unlike the zwitterionic POPC and POPC/POPS/Ca^{2+} bilayers, the lack of a ^{31}P isotropic peak from zwitterionic myelin-like membranes (Figure 6, spectrum b) suggests that mechanisms involving PE or neutral lipids such as cholesterol and cerebrosides may also contribute to the stabilization of the MBP/bilayer complexes. Recent studies support such a role for PE lipids (Fraser et al., 1989), and stable complexes of PC bilayers with MBP have been reported in the presence of cholesterol (Ter Beest & Hoekstra, 1993). Additionally, it can be noted that no spontaneous vesiculation is detected in the absence of MBP, in contrast with a previous study of myelin lipids (Fraser et al., 1986). This might be a consequence of the presence of cholesterol, missing in the myelin-like membranes of Fraser et al. (1986), which can act as a stabilizer of bilayer structures (Tilcock et al., 1982).

BIOLOGICAL RELEVANCE

In addition to membrane solubilization by detergents such as bile salts or lysophosphatidylcholine, *in vitro* disruption of phospholipid bilayers into micelles or small vesicles has been reported with biological amphipathic peptides such as cytolytic melittin.[3] Depending upon the nature of the lipid phase and its composition, melittin has been shown to induce the formation of protein-associated disk-shaped micelles or small vesicular structures upon binding to lipid bilayers (Dufourcq et al., 1986a,b). In fact, melittin-induced fragmentation of model membranes also occurs with pure phosphatidylcholine bilayers and is considerably reduced in the presence of phosphatidylserine (Dempsey et al., 1989), while bilayers of phosphatidylethanolamine remain stable (Batenburg et al., 1988). Melittin is also able to solubilize more integrated membranes, like that of the human erythrocyte, despite a high content of phosphatidylserine and phosphatidylethanolamine (Dufourcq et al., 1989). Indeed, due to the asymmetry of the lipid distribution in such membranes, the outer leaflet, which is exposed to the melittin attack, contains mainly phosphatidylcholine and sphingomyelin, while phosphatidylserine and phosphatidylethanolamine are essentially localized in the inner leaflet. Thus, it seems that a parallel can be drawn between the MBP- and melittin-induced solubilization of phospholipid bilayers. However, considering the different lengths of the melittin and MBP peptide chains (respectively 26 and 170 residues), we might expect differences in the nature, size, and lipid to protein ratio of the lipoprotein complexes obtained with these two proteins.

Demyelinating diseases such as multiple sclerosis (MS) culminate in the disruption of the myelin stacked bilayers and a complete collapse of the myelin sheath. Demyelination in MS is thought to be mediated by an autoimmune response directed against myelin antigens such as the myelin basic protein (McFarlin & McFarland, 1982; Ota et al., 1990), although this protein is located within the cytoplasmic space of myelin, shielded from the extracellular fluids by the dense lipid bilayers (Stoffel et al., 1984; Crang & Rumsby, 1990). In view of our data we can imagine that, following possible *local* defects in the lipid distribution of pathological myelin membranes (Chia et al., 1984), endogeneous MBP destabilizes the multilamellar myelin bilayers and is released from the cytoplasmic side in the form of small lipoprotein particles, with possible immune sequellae. Interestingly, a recent study which describes a peripheral nervous system demyelination induced by myelin "P_2" protein (a basic protein found in the myelin of peripheral nerves) reports that *vesiculation* was the earliest change observed in the myelin sheath, before infiltration by immune cells had occured (Rosen et al., 1992).

[2] It should be kept in mind that cholesterol, cerebrosides, and sulfatides, which may exhibit complex phase behavior (Ruocco & Shipley, 1984), do not contribute to the phosphorus spectra of these myelin lipid dispersions.

[3] Protein-induced fragmentation of lipid bilayers has also been observed with serum apolipoproteins and analogues (Brouillette et al., 1984; Anantharamaiah et al., 1985) and peptide hormone glucagon (Jones et al., 1978).

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