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Interaction of a Purified Hydrophobic Protein from Myelin with Phospholipid Membranes: Studies on Ultrastructure, Phase Transitions and Permeability

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Summary. A purified protein fraction from the proteolipids of human brain myelin was recombined with different lipids either in aqueous buffer or in a chloroform-methanol-water (10:5:1, v/v/v) mixture. It was found that under both conditions it binds strongly to phospholipids irrespective of surface charge, the presence of cholesterol or double bonds on the fatty acyl chains. The buoyant density of the resulting lipoprotein membranes is intermediate to that of pure lipids, and proteins. The lipoproteins formed by either of these methods were observed by either freeze-fracture or negative stain electron-microscopy. The overall morphology was similar to that of pure phospholipids, showing large closed multilamellar vesicles. The presence of the protein was detected by the appearance of intramembrane particles in freeze-fracture. The addition of the N-2 protein generally increases the permeability of phospholipid vesicles to $^{22}Na^+$ by 2–3 orders of magnitude depending on the concentration. The presence of calcium in the aqueous medium further increases the Na^+ efflux through negatively charged vesicles. Changes in lipid composition, surface charge, cholesterol, etc., have no appreciable influence on the effect of the protein. Differential scanning calorimetry indicates that the presence of small amounts of N-2 have no effect on the lipid phase transition from solid to liquid crystalline. As the amount of protein bound to the phospholipid increases, the enthalpy of the transition decreases, the main endothermic peak broadens, but there is no change on the midpoint temperature. Membranes containing 50% by weight of protein still show a transition with an enthalpy approximately one half that of the original lipid.

Membrane proteins can be generally categorized in two broad groups, peripheral and integral (for a recent review, *see* Singer, 1974). Interaction of the latter with membrane lipids is thought to involve nonpolar (hydrophobic) association. Evidence for such an intimate protein-lipid interaction

came from the early work of Folch and Lees (1971) which established that extraction of white matter with chloroform-methanol mixtures resulted in the solubilization of protein into the lipid solvent. The term "proteolipid" was used to designate the existence of such lipid-protein complexes. Further work showed that the proteolipid protein could be delipidized and rendered water-soluble following prolonged dialysis procedures (Tennenbaum & Folch-Pi, 1966; Folch-Pi & Stoffyn, 1972).

More recently, a protein soluble in chloroform-methanol has been isolated in highly purified form (Gagnon, Finch, Wood & Moscarello, 1971) from normal human myelin. This protein is the major component of the lipid-soluble myelin proteins. The operational name N-2 has been used for convenience[1]. It has been freed of all phosphorous-containing material, but 2% fatty acid was consistently recovered with the protein and appeared to be covalently bound (Gagnon *et al.*, 1971; Wood, Gagnon, Finch & Moscarello, 1971).

Amino acid analysis revealed that this protein contained a high proportion of apolar residues. Such composition, coupled with the firmly bound fatty acids, render it highly hydrophobic and soluble in organic solvents. However, it can also be prepared in water-soluble form after prolonged dialysis (Anthony & Moscarello, 1971), and thus it represents a versatile system for studying lipid-protein interactions. The monomer molecular weight was found to be 25,000 daltons, although when solubilized in water it aggregates to approximately 500,000 daltons (Moscarello, Gagnon, Wood, Anthony & Epand, 1973).

With the introduction of lipid vesicles as model membranes by Bangham, Standish and Watkins (1965), this system has been extensively used to study the permeability of ions (Papahadjopoulos, Nir & Ohki, 1972) and the effects of several proteins (Kimelberg & Papahadjopoulos, 1971*a*, *b*; Juliano, Kimelberg & Papahadjopoulos, 1971; Papahadjopoulos, Cowden & Kimelberg, 1973*a*; Papahadjopoulos & Kimelberg, 1973). In this report we describe the interaction of the purified myelin protein (N-2) with phospholipid vesicles (liposomes) under various conditions. The properties of the resulting membranes were studied by several techniques including Na^+ self-diffusion rates, freeze-fracture electron-microscopy and differential scanning calorimetry.

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1 *Abbreviations:* N-2, the purified main protein fraction from the proteolipid of human myelin (Gagnon *et al.*, 1971). DPPC, L-α-dipalmitoylphosphatidylcholine. DPPG, L-α-dipalmitoylphosphatidylglycerol. PS, phosphatidylserine (bovine brain). PC, phosphatidylcholine (egg yolk). TES, N-tris-(hydroxymethyl)-methyl-2-aminoethanesulfonic acid. DSC, differential scanning calorimetry.

Materials and Methods

Preparation of N-2

Myelin was isolated from human white matter by the method of Lowden, Moscarello and Morecki (1966) and N-2 extracted by the method of Gagnon *et al.* (1971). The preparation of the water-soluble form was achieved after extensive dialysis in decreasing concentrations of acetic acid as described by Anthony and Moscarello (1971). The resulting protein solution was shown to have a predominantly β-conformation (Moscarello *et al.*, 1973). This preparation was stable for several weeks.

Iodination

N-2 was iodinated by the method of Greenwood, Hunter and Glover (1963), except that free iodine was removed by prolonged dialysis instead of Sephadex chromatography. This change was necessary because the iodinated protein tended to precipitate on top of the column.

Lipids

The phospholipids used in this study were synthesized and characterized in this laboratory using the methods described in detail elsewhere (Papahadjopoulos & Miller, 1967; Papahadjopoulos, Jacobson, Nir & Isac, 1973*b*). All lipids were chromatographically pure and the fatty acid ester content was similar to that in previous studies (Papahadjopoulos & Miller, 1967). Phosphatidylcholine (PC) was extracted from egg yolk and phosphatidylserine (PS) isolated from beef brain. Dipalmitoylphosphatidylcholine (DPPC), and dipalmitoylphosphatidylglycerol (DPPG) were synthesized as described previously (Papahadjopoulos *et al.*, 1973*b*). Cholesterol (99% pure) was purchased from the Sigma Chemical Co. (St. Louis, Mo.) and recrystallized twice from methanol. All lipids were stored under nitrogen in sealed ampoules at -50 °C at a concentration of approximately 10–20 μmoles of phosphate per ml chloroform. A newly opened ampoule was used for each experiment.

Phospholipid Vesicles

Multilamellar vesicles were prepared by the method of Bangham *et al.* (1965). Unilamellar vesicles were made by sonication of multilamellar vesicles as previously described in a bath-type sonicator (Papahadjopoulos & Miller, 1967; Papahadjopoulos, 1970). The buffer used in the studies in this paper contained NaCl (100 mM or 10 mM as stated), L-histidine (2 mM), TES (2 mM), EDTA (0.1 mM) adjusted to pH 6.5 or 7.4 as stated in the text. The temperature of dispersion and sonication was either 24 °C for PS and PC or at 45 °C for DPPC and DPPG. In experiments in which unilamellar vesicles containing $^{22}Na^+$ were used, the isotope was added during the initial dispersion of the lipids in water, and the nonincorporated ions were separated by gel filtration (Papahadjopoulos, 1970) on Sephadex G-50. Self-diffusion rates were determined as before (Papahadjopoulos, 1970). The phospholipid content of vesicle preparations was measured by phosphate assay (Fiske & Subbarow, 1925) following perchloric acid digestion.

Differential Scanning Calorimetry

The transition temperature (Tc) of the phospholipids in vesicle preparations with and without protein was determined with a Differential Scanning Calorimeter (Perkin-Elmer DSC-2) using a scanning rate of 5°/min as described elsewhere (Kimelberg & Papahadjopoulos, 1974). The phospholipids were suspended in NaCl buffer or water as described in the text, at concentrations of 1–3 μmoles/ml. After incubation at specified

time and temperature, the suspensions were centrifuged for $100{,}000 \times g$ for 30 min at 24 °C, and the wet pellets transferred to the sample pan of the calorimeter. Each sample contained 0.5 to 1.0 μmoles of phosphate in 15 μliters.

The enthalpy of the transition (ΔH) was calculated from the area under each peak and the amount of phosphate present in each sample. The area was calculated by weighing the paper cut-out and converting to area from a standard curve. The phosphate was estimated by sonicating each sample after the run in 1.0 ml of 1.3% sodium deoxycholate, inside a test tube held in a bath-type sonicator until the sample pan opened and the pellet was dispersed. The calorimeter was standardized against Indium standard obtained from Perkin-Elmer. The values for ΔH obtained with purified DPPC based on the phosphate assay were within 10% of the values obtained by weighing the desiccated sample directly in the calorimeter pan, and running after equilibration with water.

Electron-microscopy

For negative stains, lightly carboned 400 mesh copper grids were pretreated with 0.01% (w/v) of bovine serum albumin and the fluid was drawn off with filter paper immediately before use. This procedure aided complete wetting of the grid. Samples were applied as drops to the grid and excess drawn off with filter paper. One drop of a 1% solution (w/v) of aqueous uranyl acetate was then added and immediately drawn off, and the grid air dried. Identical samples were also negatively stained with a 2% solution (w/v) of ammonium molybdate, pH 7.2, with essentially the same results.

For freeze-fractures the sample was made 30% v/v in glycerol and droplets of the sample (approximately 10 μliters) were mounted in gold cups at 24 °C, quickly frozen in liquid Freon 22, stored no longer than 5 min in liquid nitrogen, and mounted on a specimen table of Balzer BA-360 Freeze Etch Apparatus precooled to −150 °C. Fractures were made at −115 °C at 2×10^{-6} Torr, shadowed with C-Pt within 2 sec after the last fracture and replicated with carbon. The bell jar was then filled with dry nitrogen and replicas floated on water. Replicas were cleaned in commercial sodium hypochlorite (Javex), washed twice with water and mounted on bare 75 × 300 mesh copper grids. All specimens were examined in a Philips EM-300 operated at 60 kV using a 50 μ objective operature and the liquid nitrogen anticontamination device. An arrow in the lower left-hand corner of each freeze-fracture electron-micrograph indicates the direction of the platinum deposit. Total magnification was approximately 100,000.

Other Chemicals and Methods

L-Histidine (Sigma grade) and N-tris-(hydroxymethyl) methyl 2-aminoethane sulfonic acid (TES) were obtained from Sigma Chemical Company (St. Louis, Mo.). sodium deoxycholate was obtained from Mann Research Labs (M.A. grade). Gramicidin (a mixture of A and B) were obtained from Nutritional Biochemicals Corp. All other chemicals were reagent grade. Water was twice distilled, the second time in an all glass apparatus. Protein determinations by the method of Lowry, Rosebrough, Farr and Randall (1951), with human serum albumin as standard.

Results and Discussion

Binding of Aqueous N-2 to Preformed Vesicles

This system was used primarily for permeability studies. In this series of experiments, sonicated vesicles (Papahadjopoulos *et al.*, 1972) of phos-

phatidylserine (PS) were incubated for 1 hr at 24 °C with varying concentrations of N-2 previously iodinated and solubilized in water. The reaction medium contained NaCl (10 mM), histidine (2 mM), TES (2 mM), EDTA (0.1 mM), adjusted to pH 6.5 with NaOH. The vesicles were prepared in this buffer. Before adding N-2 the protein was dialyzed against the buffer for several hours. Both vesicles and N-2 were centrifuged at 10,000 × *g* for 30 min separately before the experiment and only the supernatants were used for mixing. Each mixture contained 4 μmoles of phospholipid and 0–2.7 mg of N-2 in 5 ml total volume. After incubation, they were centrifuged again at 10,000 × *g* for 30 min. Supernantants were separated from precipitates and both analyzed for phosphate and protein (Iodine label, ^{131}I). The results are shown in Table 1 (A).

Although PS vesicles alone or protein alone did not yield appreciable sedimentable material, the addition of N-2 produced a lipoprotein sediment of increasing protein-to-lipid ratio. From an incubation mixture containing 4 μmoles of PS and 2.8 mg protein, the resulting precipitate contained 60% protein and 40% lipid (Table 1, line 3). In this case 86% of the initial amount of protein is present in the precipitate along with 50% of the initial phospholipid. Taking into account a monomer molecular weight of 25,000 for N-2 (Moscarello *et al.*, 1973) it can be calculated that there are approximately 20 molecules of PS per N-2. Assuming 3,000 molecules of PS per vesicle (Papahadjopoulos *et al.*, 1972), the same calculation gives 150 monomer units of N-2 per PS vesicle. However, if the N-2 is still aggregated (Moscarello *et al.*, 1973) after binding to the vesicles, there would be 8 aggregate units per vesicle. (*See* Fig. 1 for freeze-fractures of such vesicles.)

Formation of Vesicles in the Presence of Aqueous N-2

In this series of experiments a solution of N-2 in water or in 10 mM buffer was added directly to the evaporated phospholipid sample, and the mixture suspended by shaking at 45 °C for 10 min. After a further incubation for 1 hr at that temperature, the mixture was centrifuged at 10,000 × *g* for 10 min and the precipitate analyzed for lipid phosphorus and protein. As shown in Table 1 (B), a considerable amount of protein was found to be bound to DPPG, the protein-to-lipid ratios being comparable to those discussed above for PS, at similar initial concentrations. However, the amount of N-2 bound to DPPC or PC membranes was smaller, the protein-to-lipid ratio being approximately 3 times lower in this case. These lipoprotein mixtures were used for either calorimetric studies or freeze-fracture electron-microscopy, as discussed below.

Table 1. Interactions of N-2 with different lipids: binding data

Method of interaction	Type of lipid	Initial mixture			Precipitate			
		Lipid (μmoles)	Protein (mg)	Volume (ml)	Lipid (μmoles)	Protein (mg)	Protein Lipid (w/w)	% by weight Protein
A	PS	4.0	0.28	5	0.5	0.15	0.39	28
	PS	4.0	0.84	5	1.0	0.70	0.87	46
	PS	4.0	2.76	5	2.0	2.36	1.5	60
B	DPPG	3	0.5	3	0.76	0.35	0.6	38
	DPPC	7	3	2	1.0	0.1	0.13	12
	PC	7	3	2	3.0	0.4	0.18	14
C	DPPC	3	2.5	2	1.22	1.07	1.15	53
	DPPG	3	1	3	1.03	0.53	0.68	40

Method A. Sonicated PS vesicles were mixed with aqueous N-2 in 10 mM NaCl buffer at pH 6.5. The mixture was centrifuged after incubation at 24 °C for 1 hr as described in the text, and the precipitate analyzed for phosphate and iodine label. Protein was calculated from the specific activity of the iodinated N-2 preparation.

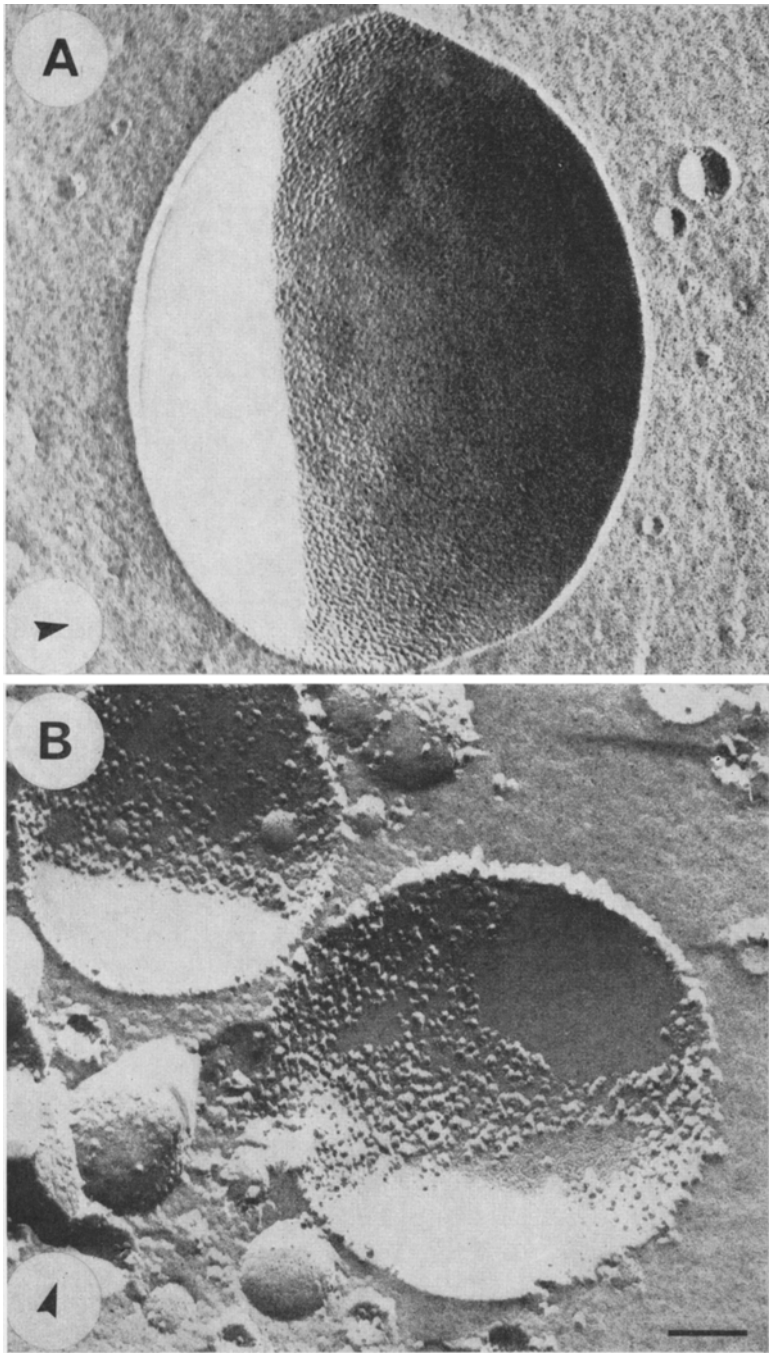
Method B. Aqueous N-2 (dialyzed against 10 mM NaCl buffer at pH 6.5) was added directly to the evaporated lipid. The mixture was suspended by mechanical (Vortex) shaking for 10 min at 45 °C and incubated at 45 °C for an additional hour. It was then centrifuged at 10,000 × *g* for 10 min at 22 °C, and the precipitate analyzed for phosphate and protein.

Method C. A certain weight of N-2 was dissolved in chloroform-methanol-water (10:5:1 volume ratio). An aliquot of phospholipid dissolved in chloroform was then added, and the mixture was evaporated to dryness under vacuum. A volume of 10 mM NaCl buffer pH 6.5 was then added, and the mixture suspended by shaking (Vortex) for 10 min at 45 °C. This was followed by additional incubation at 45 °C for 1 hr, and centrifugation at 100,000 × *g* for 30 min at 20 °C. The pellet was analyzed for phosphate and protein following centrifugation on a sucrose density gradient as described in the text.

Formation of Vesicles with N-2 Dissolved in Organic Solvent

In this series of experiments the phospholipid in chloroform solution was added to a given amount of N-2 previously dissolved in chloroform-methanol-water (10:5:1 volume ratio). The mixture was then taken to

Fig. 1. Freeze-fracture electron-microscopy of phosphatidylserine (PS) vesicles before and after addition of N-2. (*A*) 10 μmoles of PS were sonicated in 2 ml of 10 mM NaCl buffer pH 7.4. 1.3 ml of this suspension was concentrated by vacuum ultrafiltration to 0.3 ml and was used as such for freeze-fracture. As shown in the Figure, the size of the particles varies somewhat. However, most of the vesicles were of the small variety (diameter 500–1,000 Å) and only a few of larger size and presumably multilamellar



(3,000–6,000 Å diameter). (*B*) An aliquot of the original sonicated PS suspension from above (0.6 ml) containing 3 μmoles of phosphate was mixed with 0.7 ml containing 2 mg of N-2 previously dialyzed against 10 mM NaCl buffer, pH 6.5. The pellet obtained from the mixture after 10-min centrifugation in a Brinkman bench microcentrifuge was used for freeze-fracture. Bar equivalent to 1,000 Å (0.1 μm). Arrow indicates direction of shadowing

dryness under vacuum and finally suspended in water or buffer by mechanical shaking for 10 min. Each experimental mixture contained approximately 3 μmoles of lipid, 0.1–2.0 mg of N-2 and was suspended in 2 to 5 ml of final volume by shaking with glass beads. Equilibration was at 45 °C for 1 hr followed by centrifugation at 100,000 × *g* for 30 min at 20 °C. The precipitate was analyzed for phosphate and protein and was used either for calorimetry or for electronmicroscopy.

If N-2 alone was treated as above in the absence of lipids, no protein could be recovered in the aqueous suspension by simple shaking. After removal from the wall either with a pipette or with glass beads, the material appeared as large, transluscent flakes which sedimented immediately on standing. In contrast, in the presence of lipids the suspension was fairly stable on standing for at least 1 hr and considerably clearer optically than lipid alone. Mixtures of approximately equal amounts (by weight) of either egg PC or DPPC with N-2, prepared as above, were layered on discontinuous sucrose gradients and centrifuged at 100,000 × *g*. After 3 hr centrifugation the mixtures had equilibrated on top of the 40% sucrose layer (density between 1.08 and 1.17 g/ml). In parallel experiments, pure lipids were found on top of the 20% layer (density between 1.04 and 1.08 g/ml), and pure protein at the bottom of the tube, below the 40% layer (density 1.17 g/ml).

The protein and lipid analysis of such a mixture obtained from the density gradient is shown in Table 1 (C, line 2). The results indicate the presence of lipid and protein at approximately equal weights. It is thus reasonable that the mixture shows a buoyant density of less than 1.17. As will be discussed below, this mixture still exhibits a phase transition similar but broader to that of pure DPPC, and contains large vesicles.

Electron-microscopy of Phospholipid Membranes Containing N-2

As discussed above, when aqueous N-2 is added to pre-formed sonicated PS vesicles, a precipitate is formed which contains both lipids and protein. The morphology of freeze-fractures obtained with such a precipitate composed of approximately 50% protein by weight (Table 1 A) is shown in Fig. 1. A comparison between Fig. 1 *A* (PS vesicles alone concentrated by vacuum ultrafiltration) and Fig. 1 *B* (PS vesicles plus N-2) indicates that the integrity of the lipid vesicles is retained. The outstanding feature of the protein-containing vesicles is the presence of numerous particles (83 ± 8 Å diameter) which are absent from the pure lipid vesicles.

A study was also made of the morphology of DPPC vesicles formed in the presence or absence of aqueous N-2. The initial concentrations and the protein/lipid ratio of this preparation is given in Table 1 (B). These mem-

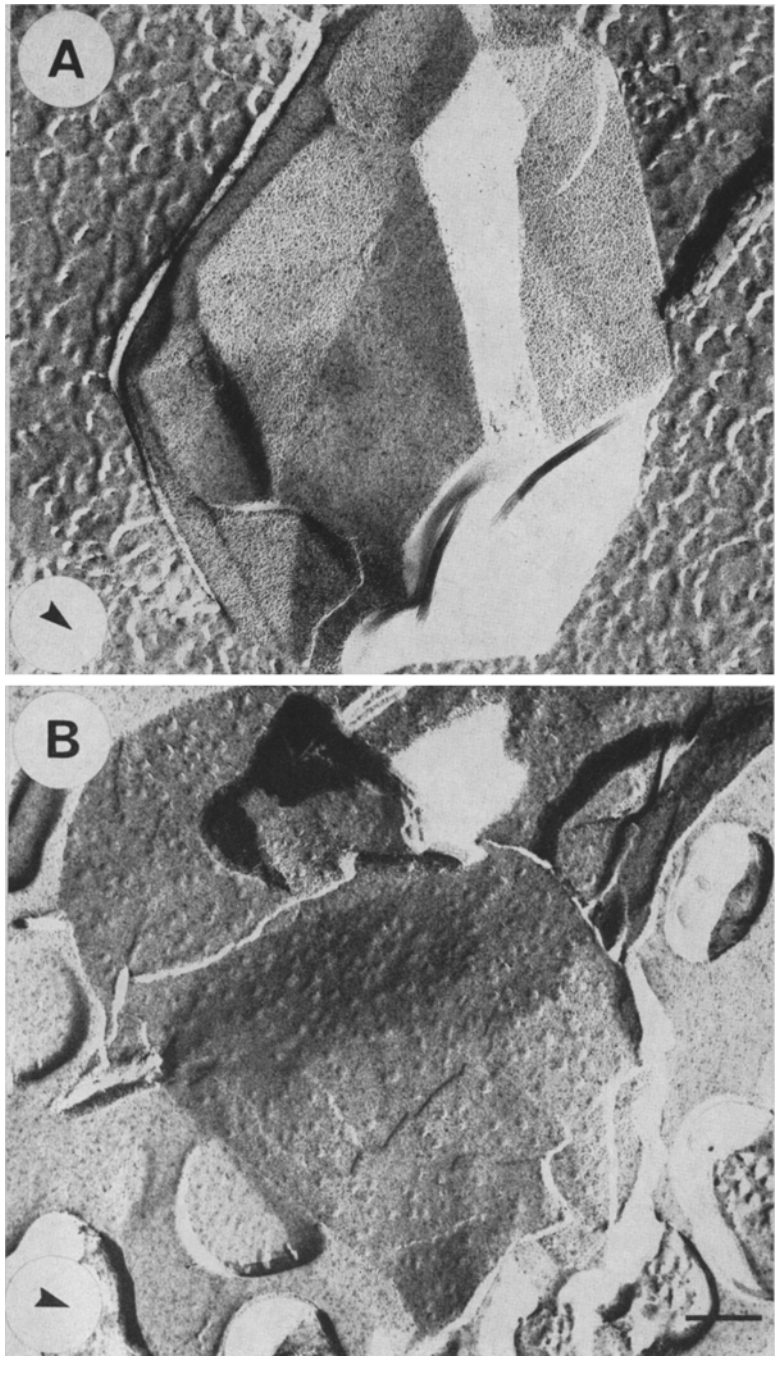


Fig. 2. Freeze-fracture electron-microscopy of dipalmitoylphosphatidylcholine (DPPC) vesicle with and without N-2. (*A*) DPPC vesicles formed by shaking the dry lipid in 10 mM buffer, pH 6.5. (*B*) DPPC vesicles formed as above, but by shaking in buffer containing N-2. Conditions and concentrations for both samples are given in Table 1 (B) and in the text. Other details as in legend to Fig. 1. Bar, 1,000 Å

branes contain approximately 10–15% protein by weight and they were made at 45 °C where DPPC is in the liquid crystalline state (above the transition temperature). However, the "quenching" to low temperatures was performed from room temperature at which the DPPC membranes would be "solid" as indicated by calorimetry (vide infra). As observed previously by other workers (Ververgaert, Verkleij, Elbers & Van Deenen, 1973), DPPC vesicles quenched from a temperature where they are solid appear to have an "angular" appearance (Fig. 2*A* and *B*). We have not observed the striated patterns reported earlier (Ververgaert *et al.*, 1973). The most interesting aspect of these pictures however, is the appearance of "particles" of approximately 110 ± 25 Å diameter in the membranes containing N-2 (Fig. 2*B*). These are particularly well defined in some DPPC membranes while others from the same preparation show no particles. In contrast, membranes of pure lipids show only smooth fractures.

Vesicles of PC and DPPC formed with N-2 dissolved in chloroform-methanol, as discussed above, also gave particles similar to those obtained when the N-2 was added in the aqueous phase. Fig. 3*A* and *B* shows freeze-fractures of egg PC vesicles without and with N-2 prepared as described in Table 1C for DPPC and contained 10% protein by weight. Numerous particles of a diameter 94 ± 12 Å can be seen clearly on the membranes containing N-2, while the vesicles of pure egg PC were smooth. Some of the particles are larger in size, appearing as aggregates of more than one. Negatively stained preparations of the same vesicles showed simply the appearance of flattened spheroidal vesicles without any particles.

In summary it can be stated that the presence of N-2, either introduced as water-soluble or lipid-soluble species, has no overall effect on the gross morphology of the phospholipid vesicles, as observed by negative staining. However, freeze-fracture of these membranes reveals the presence of intramembrane particles, not normally seen with pure lipids. The diameter of the particles varies depending on the lipid and method of recombination from 83 to 110 Å. These values include the platinum deposit, which was established in separate experiments with ferritin to average approximately 40 Å in thickness. Differences in particle density between different preparations were in general agreement with the amount of protein per total weight present in each case.

Permeability Studies

The effect of N-2 on the permeability of phospholipid membranes was studied by following the $^{22}Na^{+}$ efflux from sonicated vesicles when varying concentrations of N-2 were added to the aqueous phase. Such experiments, with three different types of vesicles are described in Fig. 4. The increase in

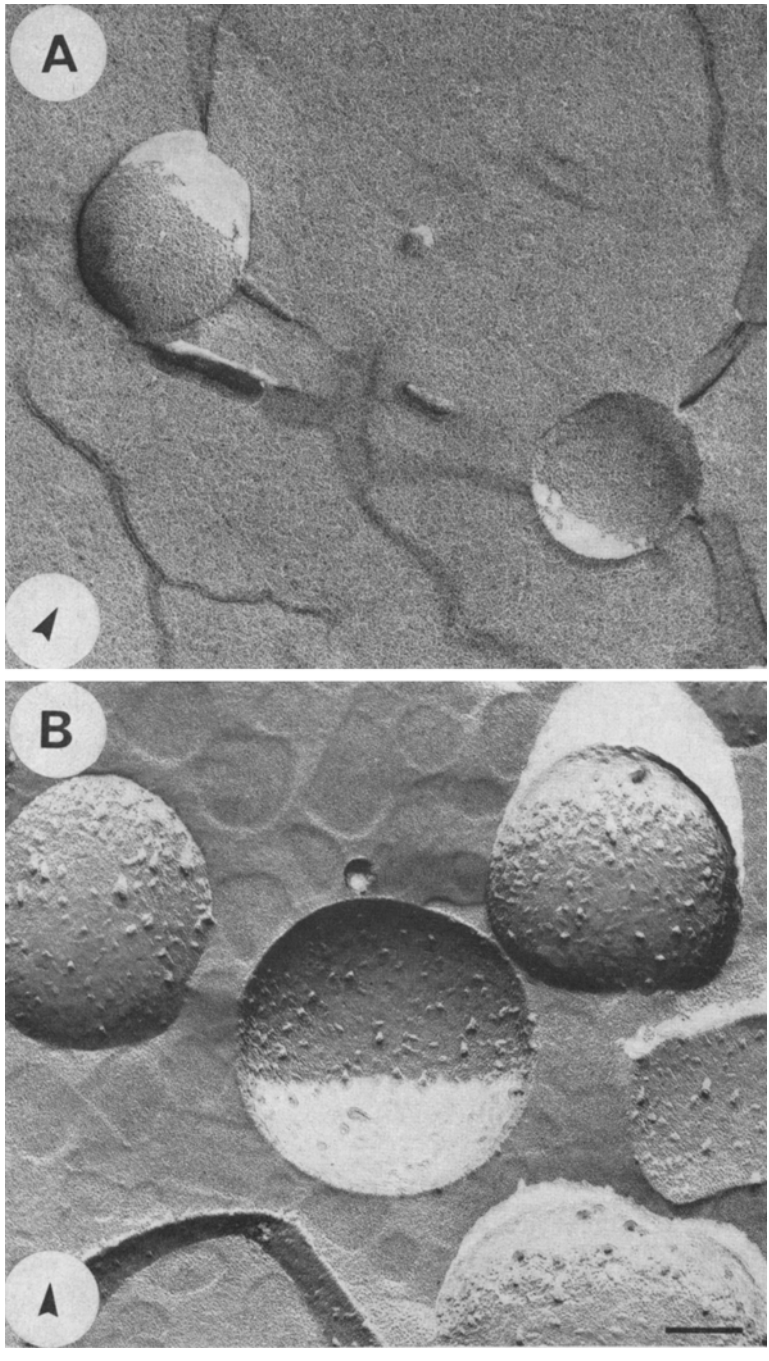


Fig. 3. Freeze-fracture electron-microscopy of phosphatidylcholine vesicles with and without N-2. (*A*) Egg phosphatidylcholine (PC) vesicles formed by shaking the dry lipid in 10 mM NaCl buffer, pH 6.5. (*B*) Egg PC was mixed with N-2 in chloroform-methanol-water (10:5:1 volume ratio). The mixture evaporated to dryness in vacuum and finally suspended in 10 mM NaCl buffer, pH 6.5 at 45 °C. The pellet obtained by centrifugation of each preparation was used for freeze-fracture as described in Materials and Methods. Other details as in Fig. 1. Bar, 1,000 Å

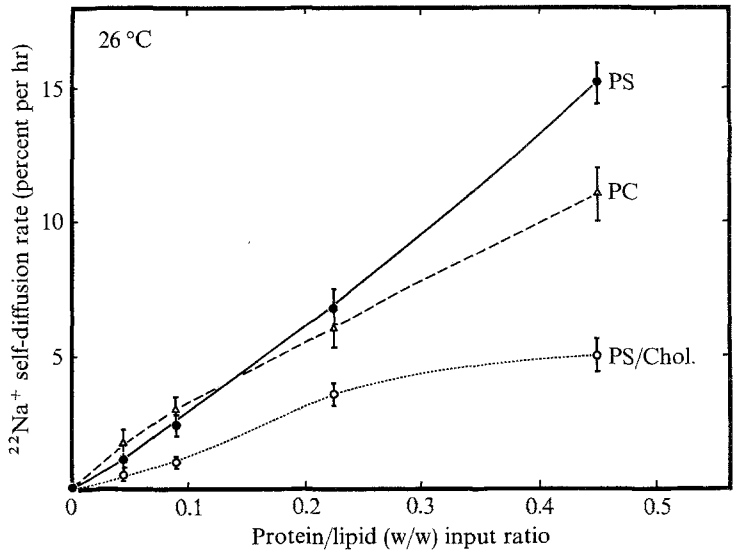


Fig. 4. Effect of N-2 on permeability of phospholipid vesicles. Sonicated vesicles of phosphatidylserine (PS), phosphatidylcholine (PC) and equimolar mixtures of phosphatidylserine with cholesterol (PS/Chol) were made to contain $^{22}Na^+$, in 10 mM NaCl buffer at pH 6.5. Different amounts of N-2, previously made water-soluble and dialyzed against the same buffer, were added to aliquots of lipid vesicles containing 1 μmole of phosphate in 1 ml final volume. The mixtures were dialyzed against 10 ml of the same buffer at 26 °C, and the presence of $^{22}Na^+$ in the dialysate measured at 1-hr intervals. The self-diffusion rate of $^{22}Na^+$ was expressed as a percent of the $^{22}Na^+$ present inside the vesicles at zero time. The results shown were obtained during the first hour of dialysis. The diffusion rates were lower by approximately 50% during the second hour

$^{22}Na^+$ self-diffusion rate is approximately linear up to protein/lipid ratios of 0.45. At this point the increase in Na^+ efflux through PS vesicles is approximately 300-fold (control without protein 0.05% per hr). From the binding data discussed earlier (Table 1) it appears that the weight ratio of protein to lipid is approximately 1 at this point. The effect on permeability and the amount of protein bound per mg lipid are similar to those reported earlier for cytochrome *c* (Kimelberg & Papahadjopoulos, 1971*b*), although much higher concentrations of cytochrome *c* in the aqueous phase were required for the same effect (20 mg cytochrome *c* per mg PS).

The effect of the N-2 on the permeability of the PC membranes was similar to that found with PS indicating that the surface charge is not of primary importance for the interaction. The effect of N-2 on vesicles containing PS mixed with equimolar amounts of cholesterol was approximately one-third to that with pure PS vesicles (Fig. 4). However, the presence of equimolar amounts of cholesterol reduces the Na^+ permeability of the pure

lipid vesicles to the same degree (Papahadjopoulos *et al.*, 1972). These results taken together show that the N-2 produces an equivalent increase in Na^+ efflux through PS and PS/cholesterol membranes, indicating that cholesterol does not inhibit the interaction. This is in contrast to results reported recently (Papahadjopoulos *et al.*, 1973*a*) in which the presence of cholesterol generally inhibited the ability of several other proteins to increase the permeability of phospholipid membranes. It should be pointed out that aggregation and settling-out of lipoprotein membranes was observed with all three types of vesicles within 1 hr following addition of N-2 in the bulk phase.

In all cases described in Fig. 4 the vesicles were made and incubated with protein in 10 mM buffer, pH 6.5. Change of the bulk-phase pH to 4.5 or to 8.5 subsequent to the addition of the protein had no appreciable effect on the Na^+ efflux rate. Increasing the ionic strength by increasing NaCl to 100 mM showed a slight increase in permeability. On the other hand, addition of Ca^{2+} to a concentration of 0.5 mM had a large effect for the PS vesicles containing N-2 (fourfold stimulation of $^{22}Na^+$ efflux over control), but none for the PC vesicles. This synergistic effect of Ca^{2+} was also found earlier with spectrin and PS vesicles (Juliano *et al.*, 1971). It appears from the above studies that the ability of N-2 to increase the permeability of phospholipid membranes is not dependent on surface charge, changes in pH (between 6.5 and 8.5), ionic strength (between 0.01 and 0.1) or the presence of cholesterol at equimolar ratio. However, the effect can be greatly augmented by the presence of small amounts of Ca^{2+}, if the membranes are negatively charged.

Calorimetric Studies

An important parameter of lipid-protein interactions is the effect of a given protein on the thermotropic transition of lipid membranes. To investigate the effect of N-2, we have studied the endothermic transition of DPPC and DPPG in the presence of varying amounts of N-2, with a Differential Scanning Calorimeter. The N-2 was added to the phospholipids either solubilized in water or after solubilization in chloroform-methanol. The resulting mixed dispersions either in water or 10 mM NaCl buffer were usually incubated at 45 °C for 1 hr and the pellet obtained by centrifugation was used as a sample for the calorimeter and subsequently analyzed for phosphate and protein content.

Fig. 5 shows some of the calorimetric data obtained with pure DPPC (Curve 1) and increasing amounts of N-2 in DPPC (Curves 2 to 5). It is

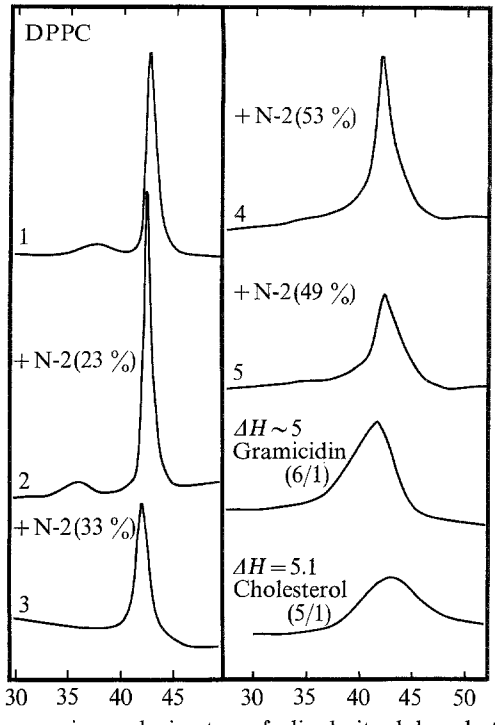


Fig. 5. Differential scanning calorimetry of dipalmitoylphosphatidylcholine (DPPC) membranes containing N-2. Every mixture contained 3 μmoles of DPPC mixed in chloroform-methanol-water with different amounts of N-2 which was added as solid powder to this solvent. Each mixture was suspended in 2 ml of 10 mM NaCl buffer, pH 6.5 at 42 °C for 1 hr with shaking by Vortex mixer. The suspensions were then centrifuged for 10 min at room temperature in the Brinkman bench-top microcentrifuge. The wet pellets were transferred to the calorimeter sample pans with a pasteur pipette. Each sample was analyzed for phosphate and protein after calorimetry. The amount of phosphate usually present was 1 μmole. Curve 1: pure DPPC. Curve 2: DPPC mixed with 0.72 mg N-2. Final amounts in the sample 1.12 μmoles phosphate and 0.24 mg protein. Curve 3: DPPC mixed with 1.1 mg N-2; final amounts: 1.1 μmoles DPPC and 0.42 mg protein. Curve 4: 3 μmoles DPPC mixed with 2.4 mg N-2 and suspended in 2 ml of distilled water. The suspension was centrifuged on a discontinuous sucrose gradient. The material collected at the interface between 20 and 40% sucrose was resuspended in water and the pellet after cenrifugation transferred to the calorimeter sample pan. Final amounts: 1.22 μmoles phosphate and 1.1 mg protein. Curve 5: DPPC mixed with 2.42 mg N-2 as above and suspended in 2 ml of buffer, pH 6.5. Final amounts in sample: 1.23 μmoles phosphate and 0.9 mg protein. Curve Gramicidin: 6 μmoles of DPPC were mixed with 1 μmole Gramicidin A in chloroform-ethanol (1:1 by volume) evaporated to dryness, and suspended in 0.1 ml of 100 mM NaCl buffer, pH 7.4 at 45 °C. Curve Cholesterol: 2.5 μmoles of DPPC were mixed in chloroform with 0.5 μmoles of cholesterol, evaporated to dryness and suspended in 2 ml of 100 mM NaCl buffer, pH 7.4 at 42 °C

apparent that although the mid-point of the main endothermic peak is constant (42 °C), the peak becomes increasingly broader, while the pre-melt

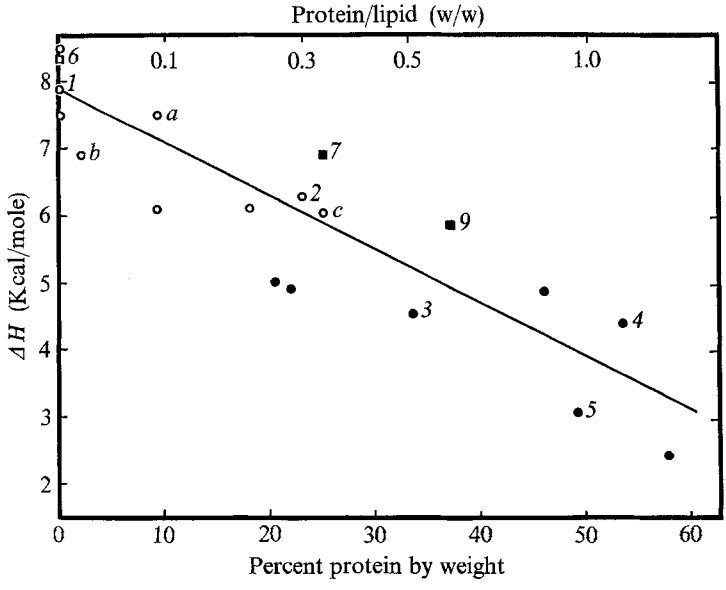


Fig. 6. Enthalpy of transition for the melting of phospholipid membranes containing different amounts of N-2. The enthalpy of transition (ΔH) was obtained from the area under the main endothermic peak obtained with each sample, and the phosphate present. Percent protein by weight was calculated from the weight of the lipid obtained from the phosphate assay assuming a molecular weight of 760 and the weight of the protein by the Lowry assay. Open and closed circles represent data obtained with DPPC membranes. Squares, data obtained with DPPG membranes. Numbers refer to the numbering of the curves in Fig. 5, except for numbers 6, 7 and 9 obtained with DPPG, which are not shown as separate curves (*see text*). The preparation of samples was as described in Fig. 5 except for the following. Point *a*: 3 μmoles of DPPC suspended in 3 ml of water containing 0.5 mg N-2 (α-conformation). Point *b*: 3 μmoles of DPPC suspended in 3 ml of water containing 0.5 mg N-2 (β-conformation). α and β conformations of N-2 prepared as described by Moscarello *et al.* (1973). Point *c*: 4 μmoles of DPPC was suspended in 1 ml of water containing 4 mg N-2

transition disappears from samples containing more than 20% protein by weight. For purposes of comparison, Fig. 5 also depicts the calorimetric characteristics of a mixture of DPPC with gramicidin (6:1 molar ratio) and DPPC with cholesterol (5:1 molar ratio).

The enthalpy of the transition, as obtained from the samples already discussed and others not shown, is plotted in Fig. 6 against the percentage of protein (by weight) in each sample. Pure DPPC gives values of enthalpy (ΔH) of approximately 8 kcal/mole. Samples with increasing percentage of N-2 exhibit lower values of ΔH, which decreases to approximately 4 kcal/mole for mixtures containing 50% N-2 by weight. Although there is consider-

able spread between points, the general trend seems to be a linear decrease of ΔH as a function of protein concentration.

It is of interest to note that the amount of cholesterol present in the sample included in Fig. 5 is only 9% by weight. However, its effect in terms of peak broadening and decrease in ΔH is comparable to that produced by 50% (w/w) N-2 in DPPC membranes. It is also worth noting that for mixed membranes containing N-2 up to 20–25% by weight, there is no overall effect on the shape of the calorimetric curve. The samples which gave a sharp major transition and a pre-melt are depicted with open circles in Fig. 6. The numbering of the curves in Fig. 5 corresponds to the numbering of the points in Fig. 6, and the conditions for the preparation of each sample are given in the legend to Fig. 6.

The interaction of N-2 with a negatively charged phospholipid (DPPG) was also studied to determine the effect of surface charge and ionic strength on the interaction with N-2. Pure DPPG dispersed in 100 mM NaCl solutions at pH 7.4 exhibits thermotropic transitions similar to those of DPPC (Jacobson & Papahadjopoulos, 1975). The main peak is slightly broader than the one of DPPC, with a mid-point at 41 °C and a well-defined pre-melt. DPPG dispersed in 10 mM buffer shows a slightly broader transition with a very small pre-melt. When N-2 was added during dispersion in both 10 mM and 100 mM NaCl solutions, the overall effect was broadening of the main peak, loss of the pre-melt and a decrease in the ΔH, similar to that observed with DPPC. The points numbered 6, 7 and 9 (squares) in Fig. 6 were obtained with DPPG. It appears then that N-2 has similar effects on the thermotropic transition of DPPG and DPPC, and these effects are not affected by ionic strength, indicating that the predominant bonds are nonionic.

Discussion

The protein used in this study is the major component of the proteolipid protein from human myelin. More than 60% of the amino acid residues are apolar in addition to the presence of 2% fatty acids. It is tempting to speculate that the apolar residues are clustered at one end of the molecule, forming a core which can be readily accommodated in a lipid phase. Structural studies are under way to determine if in fact the molecule does contain such an apolar region as shown recently for the sialoprotein isolated from erythrocytes (Marchesi, Tillack, Jackson, Segrest & Scott, 1972). In any case, the protein used in this study has been shown to exhibit certain "conformational flexibility" in assuming a predominantly α-helical or β-conformation

depending on the dialysis procedure used to solubilize it in water (Moscarello *et al.*, 1973).

The interactions between this "hydrophobic" membrane protein (N-2) and lipid vesicles affords an interesting system for the study of lipid-protein interactions and membrane structure. By the use of freeze-fracture techniques it can be shown that lipid vesicles give smooth fracture surfaces. When the N-2 protein is incorporated into the vesicles the fracture surfaces contain particles which presumably represent areas where protein has been incorporated into the lipid bilayer. Intramembrane particles were also observed when purified rhodopsin was incorporated into lipid bilayers (Hong & Hubbell, 1972). In addition to its effect on the morphology, the presence of protein drastically alters the permeability of phospholipid vesicles as judged by the increase in $^{22}Na^{+}$ efflux (Fig. 4). Evidence for a correlation between such increase in permeability and the ability of proteins to "penetrate" into phospholipid monolayers has already been presented (Kimelberg & Papahadjopoulos, 1971*a*, *b*).

Monolayer studies have shown that the addition of the N-2 protein in the water phase tends to increase the film pressure and results in area expansion when the lipid monolayer is held at constant pressure (Papahadjopoulos *et al.*, 1973*a*). Penetration of total proteolipid protein into various lipid monolayers was also reported recently (London, Demel, Geurts Van Kessel, Zahler & Van Deenen, 1974). Since the calorimetric and permeability properties were not affected by changes in ionic strength and pH, or the presence of surface charge, the interactions involved are non-ionic, and probably hydrophobic. From all the available evidence so far we conclude that the N-2 protein is at least partly embedded into the lipid phase. Thus, its localization within the lipid membrane in the reconstituted systems could be similar to that proposed for the other "intrinsic" membrane proteins (Vanderkooi & Green, 1970; Singer & Nicolson, 1972). Whether this protein penetrates only one-half of the lipid bilayer or whether it spans the bilayer cannot be answered at present.

It should be made clear at this point that our conclusions concerning the localization and interactions of the purified N-2 protein with the lipid membrane in the reconstituted systems are not directly applicable to the structure of the native myelin. Several important points have yet to be answered before such comparison can be made. For example, myelin membranes have been shown to have smooth fracture surfaces, with very few, if any, intramembrane particles (Branton, 1967). The appearance of such particles in our reconstituted membranes raises the question as to whether these particles are artefacts of the protein preparation and method

of reconstitution. Related to the above is the question as to whether the conformation of N-2 in the reconstituted membranes is similar to that of its native state. Our studies at this point cannot answer adequately the above questions, but the following points are relevant: (1) The concentration of N-2 in human brain myelin constitutes only 6% of the total dry weight (Gagon *et al.*, 1971) and thus it is present at much lower concentrations compared to our reconstituted membranes. (2) Freeze-fractures of purified myelin from human white matter, which was used for the isolation of the N-2 protein, do show a low density of intramembrane particles 80–90 Å diameter (Vail & Moscarello, *in preparation*). (3) Labeling of N-2 in reconstituted membranes with the reagent DIDS (4,4′-diisothiocyano-2,2′-dihydrostilbene disulfonate) indicates a specific reactivity among several peptide fragments of the protein, similar to that obtained with native myelin, indicating a possible similarity of the N-2 protein localization within the lipid environment in native and reconstituted systems (Moscarello & Papahadjopoulos, *in preparation*).

The effects of the N-2 protein on the thermotropic phase transition of the lipid membranes is of considerable interest. The results obtained by the differential scanning calorimeter with DPPC and DPPG membranes in the presence of different amounts of N-2, indicated that the protein has no appreciable effect on the temperature of the mid-point of the transition. However, the transiton occurs over a wider temperature range and the enthalpy is reduced in proportion to the amount of protein present. From the data shown in Fig. 6, it can be concluded that for lipoprotein membranes containing equal amounts of lipid and protein by weight, only half of the lipid chains are participating in the cooperative transition from a solid to a fluid bilayer. By inference, the rest of the lipid is bound to the protein with a ratio of 0.5 (w/w) lipid per protein. A ratio of 0.2 (w/w) bound lipid per protein was arrived at recently in a study of cytochrome oxidase-lipid complexes by spin-labels (Jost, Griffith, Capaldi & Vanderkooi, 1973). In agreement with the above study, we conclude that the N-2 protein binds a certain amount of lipid within the bilayer, but it leaves the rest of the bilayer largely "unperturbed". Assuming a molecular weight of 25,000 for the monomeric form of N-2 (Moscarello *et al.*, 1973) there would be approximately 15 phospholipid molecules bound to each protein monomer in the above mixtures.

In contrast to the above results, we have found that the other major protein component from myelin, the positively charged encephalitogenic component (Eylar, Brostoff, Hashim, Caccam & Burnett, 1971) behaves quite differently. Thus, when allowed to react with DPPG membranes, the

basic myelin protein not only broadens the transition temperature range and reduces the ΔH, it also reduces the mid-point temperature by 10 °C. These results indicate that the bulk phospholipid membrane is "fluidized" by the protein (Papahadjopoulos, Moscarello & Eylar, *in preparation*). This is similar to the effects reported for cytochrome *c* on mycoplasma phospholipids (Chapman & Urbina, 1971) and also on PS membranes (Chapman, Urbina & Keough, 1974). We interpret these effects as the result of partial "penetration" and "deformation" of the bilayer by these proteins (Kimelberg & Papahadjopoulos, 1971*a*; Papahadjopoulos *et al.*, 1973*a*). Other positively charged proteins, such as ribonuclease have no effect on the mid-point temperature, and poly-L-lysine actually produces an increase on the temperature of transition of DPPG (Papahadjopoulos, *unpublished data*). This indicates a stabilization of the lipid bilayer, and correlates well with previous data indicating lack of penetration into lipid monolayers and no effects on the permeability of the lipid bilayers (Kimelberg & Papahadjopoulos, 1971*a*, *b*). Such stabilization of dilaurylphosphatidylglycerol by the basic myelin protein was reported recently (Verkleij *et al.*, 1974). The different types of lipid-protein interactions as defined by monolayer penetration and vesicle permeability were recently reviewed (Papahadjopoulos & Kimelberg, 1973).

The results presented in this paper provide useful information relating to the thermotropic behavior of the mycoplasma and other biological membranes. As shown by the studies of Steim and colleagues (Steim, Tourtellotte, Reinert, McElhaney & Rader, 1969; Blazyk & Steim, 1972) the thermotropic transition of mycoplasma and also mitochondria membranes had a similar mid-point temperature to that of the extracted lipids from the same membranes suspended in buffer. Moreover, the enthalpy of the lipid transition in these membranes was 75 to 80% of that observed for the isolated lipids. It was concluded from these results that the majority of the lipids in these membranes associate with each other as in a pure bilayer. However, this still leaves open the question of protein localization since it is not known how each type of lipid-protein interaction would affect the thermotropic properties of the lipids. The results described earlier in this paper indicate clearly that relatively large amounts of protein can interact through nonionic bonds and probably penetrate into the bilayer without large distortion of the thermotropic phase transition, as observed by DSC. Furthermore, ionically bound proteins can have a large "fluidizing" effect, or a "stabilizing" effect depending on the degree of nonpolar interactions and penetration into the bilayer. It is of interest to note that of the two main myelin proteins discussed here, the "hydrophobic" N-2 produces

considerably smaller changes on the lipid phase transition, compared to the water-soluble basic myelin protein. Finally, it should be emphasized that divalent metals can also play an important role in the thermotropic properties of membranes, by generally increasing the stability of acidic phospholipid bilayers (Kimelberg & Papahadjopoulos, 1974; Trauble & Eibl, 1974; Verkleij *et al.*, 1974; Jacobson & Papahadjopoulos, 1975).

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