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EFFECTS OF PROTEINS ON THERMOTROPIC PHASE TRANSITIONS OF PHOSPHOLIPID MEMBRANES

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SUMMARY

A variety of proteins have been studied for their ability to interact and alter the thermotropic properties of phospholipid bilayer membranes as detected by differential scanning calorimeter. The proteins studied included: basic myelin protein (A1 protein), cytochrome *c*, major apoprotein of myelin proteolipid (N-2 apoprotein), gramicidin A, polylysine, ribonuclease and hemoglobin. The lipids used for the interactions were dipalmitoylphosphatidylcholine and dipalmitoylphosphatidylglycerol. The interactions were grouped in three categories each having very different effects on the phospholipid phase transition from solid to liquid crystalline. The calorimetric studies were also correlated with data from vesicle permeability and monolayer expansion.

Ribonuclease and polylysine which exemplify group 1 interactions, show strong dependence on electrostatic binding. Their effects on lipid bilayers include an increase in the enthalpy of transition (ΔH) accompanied by either an increase or no change in the temperature of transition (T_c). In addition, they show minimal effects on vesicle permeability and monolayer expansion. It was concluded that these interactions represent simple surface binding of the protein on the lipid bilayer without penetration into the hydrocarbon region.

Cytochrome *c* and A1 protein, which exemplify group 2 interactions, also show a strong dependence on the presence of net negative charges on the lipid bilayers for their binding. In contrast to the first group, however, they induce a drastic decrease in both T_c and ΔH of the lipid phase transition. Furthermore, they induce a large increase in the permeability of vesicles and a substantial expansion in area of closely packed monolayers at the air-water interface. It was concluded that group 2 interactions represent surface binding followed by partial penetration and/or deformation of the bilayer.

Group 3 interactions, shown by proteolipid apoprotein and gramicidin A,

Abbreviations: TES, *N*-tris-(hydroxymethyl)-methyl-2-aminoethane sulfonic acid; T_c, phase transition mid-point temperature; ΔH, transition enthalpy; A1 protein, basic myelin protein; N-2 apoprotein, the major apoprotein of myelin proteolipid.

were primarily non-polar in character, not requiring electrostatic charges and not inhibited by salt and pH changes. They had no appreciable effect on the T_c but did induce a linear decrease in the magnitude of the ΔH, proportional to the percentage of protein by weight. Membranes containing 50% proteolipid protein still exhibited a thermotropic transition with a ΔH one half that of the pure lipid, and only a small diminution of the size of the cooperative unit. It was concluded that in this case the protein was embedded within the bilayer, associating with a limited number of molecules via non-polar interactions, while the rest of the bilayer was largely unperturbed.

INTRODUCTION

Considerable current research interest focuses on the role of phospholipid phase transitions on transport properties of cell membranes [1–4] and lipid-dependent membrane enzymes [5–10]. The subject is of general biological interest since lipid fluidity could be affecting many aspects of cell behavior [11–18].

Intimately related to the above subject is the question as to how different proteins affect lipid fluidity and consequently the thermotropic phase transitions of phospholipid bilayers with which they might interact. The calorimetric studies of Steim and colleagues [19, 20] have established that there is a general similarity between the thermotropic transitions of intact membranes from mycoplasma, mitochondria and microsomes and those of the extracted lipids when suspended in aqueous salt solutions. This overall similarity was also noted with *Escherichia Coli* membranes and extracted lipids using fluorescence techniques [21]. The conclusion drawn from the above studies was that the large majority of membrane lipids associate with each other as in a pure bilayer.

The interpretation of such studies is complicated by the present lack of detailed information concerning the effects of different types of lipid-protein interactions on the thermotropic properties of the lipids. The available information indicates that a variety of effects could be expected. For example Chapman and colleagues [22, 23] noted that cytochrome *c* decreases the transition temperature (T_c) of phosphatidylglycerol membranes, while Verkleij et al. [24] have reported that the basic myelin protein increases the T_c of similar membranes. Inhibition in molecular motion of spin probes was reported to be produced by rhodopsin on lecithin membranes [25] and also by cytochrome oxidase on a fraction of the lipid molecules in reconstituted systems [26]. Various effects on the orientation of lipid molecules were also reported by Butler et al. [27].

In this report, we have examined the effects of several proteins and peptides on the thermotropic properties of highly purified dipalmitoylphospholipids as detected by a differential scanning calorimeter. These results were then correlated with the effects of the same proteins on the permeability of phospholipid vesicles and the expansion of phospholipid mono-molecular films at the air-water interface. It was concluded that the effect of a particular protein on the T_c and the enthalpy of transition depends on the type of interaction and the degree of "penetration" into the bilayer. The proteins studied here were grouped in three broad types of interactions: (1) surface adsorption, (2) surface adsorption followed by partial penetration and deformation of bilayer, (3) complete penetration of the protein molecule (or part of it) into the bilayer.

MATERIALS AND METHODS

Proteins. The major apoprotein from myelin proteolipid (N-2 apoprotein) was purified from human brain myelin [28] and was solubilized in water following extensive dialysis in decreasing concentrations of acetic acid [29]. The resulting preparation in aqueous solution was shown to have a predominantly β-conformation [30] and an aggregate weight of 500000 daltons, while the monomer appears to be 25000 daltons [30]. The basic myelin protein (A1 protein) was purified from bovine brain [31]. The complete amino acid sequence has been determined [32], and the molecular weight estimated as 18 400 [33], with a conformation devoid of α-helical and β-structures, and high intrinsic viscosity [33, 34]. Cytochrome *c* (horse heart, Type VI) was obtained from Sigma; Ribonuclease (bovine pancreas, RAF type) from Worthington. Albumin (human serum, crystallized) from Miles-Pentex; hemoglobin (human, twice crystallized) from Mann Research; poly-L-lysine (mol. wt 17000) from Mann, Gramicidin A (activity 100%) from Nutritional Biochemicals. All proteins and peptides obtained commercially were used without further purification, except for a preliminary dialysis against the buffer.

Lipids. All phospholipids used in this study were synthesized and characterized in this laboratory using methods described in detail elsewhere [35] and were chromatographically pure. Dipalmitoylphosphatidylcholine (1,2-dihexadecyl-*sn*-glycero-3-phosphoryl-choline) was synthesized by re-acylation of egg yolk lecithin [36] by the method of Robles and Van den Berg [37]. Dipalmitoylphosphatidylglycerol was synthesized by a minor modification [35] of the method of Dawson [38]. All lipids were stored in chloroform under N_2 in sealed ampoules at −50 °C at concentration of approx. 10 μmol/ml. Each ampoule was newly opened for each experiment.

Phospholipid vesicles were prepared by evaporating the chloroform solution under vacuum and suspending the material in aqueous buffer containing NaCl (100 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. Suspension was accomplished by shaking in a vortex mixer under N_2 for 10 min at 45 °C. The suspensions (containing 3 μmol phosphate in 1.5 ml) were equilibrated at 45 °C for 1 h, and then centrifuged at 10 000 × *g* for 10 min at room temperature. The resulting wet pellets were used immediately for calorimetry. In some cases, as stated in the text, the initial suspensions were sonicated in a bath-type sonicator, inside a closed tube under N_2 for 1 h at 45 °C as described before [39]. The sonicated suspensions consisting mostly of small vesicles (less than 2 000 Å diameter as judged by negative stain electron microscopy) were concentrated by ultrafiltration at room temperature before calorimetry. Self-diffusion rates of $^{22}Na^+$ through sonicated vesicles were determined as before [40].

Interaction with proteins. Proteins and peptides were brought in contact with the lipids by one of three methods as stated in the text. One: The protein was dialyzed against the desired buffer and was added to the dry lipid during the initial suspension. The preparation was then treated as the pure lipids described above. Two: The protein (N-2, the proteolipid apoprotein) was dissolved in chloroform/methanol/water (10-5-1, v/v). Gramicidin was dissolved in ethanol. In either case, the lipid was added in chloroform solution and the mixture evaporated to dryness under vacuum. Finally, it was suspended in buffer by vortex shaking for 10 min at 45 °C and treated as above. Three: Protein dialyzed against the desired buffer was added to pre-formed sonicated

vesicles and incubated at the stated temperature for 1 h, centrifuged at 10 000 · *g* at room temperature for 10 min and the pellet used for calorimetry. The amount of lipid in each preparation was 3 μmol of phosphate in 1.5 ml to 5 ml total volume and protein concentration as stated.

Monolayer area expansion under constant pressure, induced by proteins added to the subphase, was determined as before [42]. Protein was determined by the method of Lowry et al. [43] with human serum albumin as standard. Phosphate was determined by a variation [6] of the Fiske and SubbaRow method [44].

Differential scanning calorimetry. A Perkin-Elmer DSC-2 was used throughout with a scanning rate of 5 °C per min as described elsewhere [41]. Only heating scans are reported throughout the paper and they were found to be reproducible upon reheating unless specifically stated otherwise. The sensitivity range was usually 1 mCal/s for full scale (10 inch) displacement. The samples were obtained from the wet pellets after centrifugation (10 000 · *g* for 10 min at 24 °C) of the suspensions containing lipids with and without different proteins. Each sample contained 0.5–1.0 μmol of phosphate in 10–15 μl total volume. Phosphate and protein content were determined after calorimetry, by sonicating each sample 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 ob-

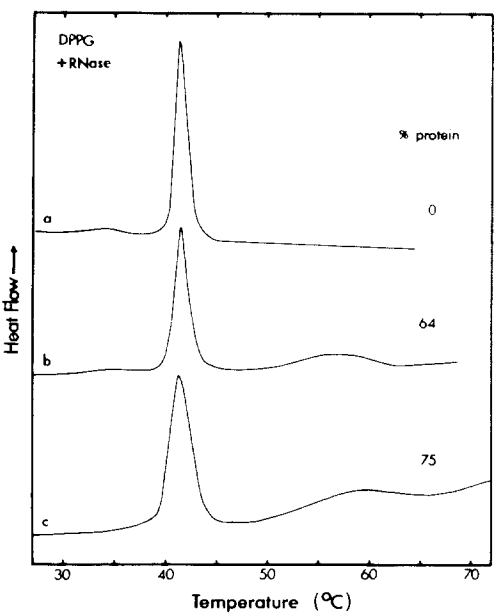

Fig. 1. Differential scanning calorimetry of dipalmitoylphosphatidylglycerol (DPPG) membranes prepared in the presence of ribonuclease. Each of the mixtures contained 3 μmol of dipalmitoylphosphatidylglycerol with or without added protein in 1.5 ml total volume of 10 mM NaCl buffer at pH 7.4. a, dipalmitoylphosphatidylglycerol alone; 2.1 μmol of phosphate in pellet; b, dipalmitoylphosphatidylglycerol plus 10 mg ribonuclease; 1.2 μmol phosphate and 1.6 mg protein in pellet; c, dipalmitoylphosphatidylglycerol plus 30 mg of ribonuclease; 0.95 μmol phosphate and 2.25 mg protein in pellet. The arrow on the left margin of this and the following figures indicates the direction for an endothermic transition.

tained from Perkin-Elmer. The enthalpy of transition was obtained from the area under each peak and the amount of phosphate in each sample. The area was calculated by weighing the paper cut-outs and converting to areas from standard curve. The mid-point of each peak was defined as the T_c. Reduction of the heating rate from 5 to 1.25 °C per min had a small but consistent effect in all samples, producing a decrease in the mid-point T_c by 1 °C. Each experiment was repeated at least three times with consistent results.

Other chemicals. L-Histidine (Sigma grade) and *N*-tris-(hydroxymethyl)-methyl 2-aminoethane (TES) were obtained from Sigma Chemical Co. (St. Louis, Mo.). Sodium deoxycholate from Mann Research Labs (M.A. grade). All other chemicals were reagent grade. Water was twice distilled, the second time in an all glass apparatus.

RESULTS AND DISCUSSION

Interactions with ribonuclease and polylysine (group 1)

Different amounts of either pancreatic ribonuclease and poly-L-lysine were added to dipalmitoylphosphatidylglycerol in 10 mM NaCl aqueous solutions at pH 7.4, and the mixture suspended together by shaking at 45 °C, as described for interaction method "one" in Materials and Methods. The results are shown in Figs 1 and 2.

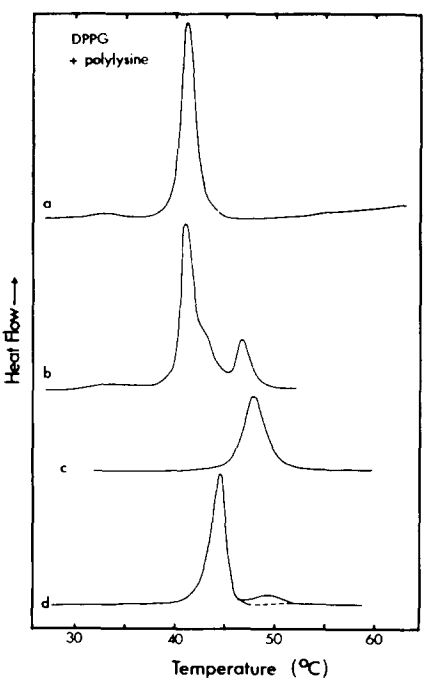

Fig. 2. Differential scanning calorimetry of dipalmitoylphosphatidylglycerol (DPPG) membranes prepared in the presence of polylysine. Each mixture contained 3 μmol of dipalmitoylphosphatidylglycerol in 1.5 ml of 10 mM NaCl buffer at pH 7.4, with different amounts of polylysine present: a, none; b, 0.2 mg; c, 0.8 mg; d, 4 mg.

As reported earlier [41] dipalmitoylphosphatidylglycerol undergoes an endothermic transition with a main peak mid-point at 41.5 °C and a pre-melt point at 34 °C (curve a Fig. 1).The pellets obtained in the presence of 10 and 30 mg ribonuclease (curve b and c, respectively, of Fig. 1) exhibited also a main endothermic peak with a mid-point at 41.5 °C. However, the pre-melt peak was either diminished (10 mg) or absent (30 mg) and the main peak was considerably broadened. The excess heat associated with the main transition (at 41.5 °C) was increased from 7.9 ± 0.4 kcal/mol for phosphatidylglycerol alone, to 8.9, 9.5 and 9.7 kcal/mol in the presence of 5, 10 and 30 mg of ribonuclease, respectively.

The amount of ribonuclease protein associated with the pellets is expressed as a percentage of the total weight of lipid plus protein present and was found to be 64 and 75 % for the two samples shown in Fig. 1. The shallow peak appearing with a mid-point at 57 °C in the samples containing proteins probably corresponds to the thermal transition of ribonuclease associated with the lipid. Concentrated solutions of ribonuclease alone show an endothermic peak with a mid-point at 64 °C. It appears then that the binding of ribonuclease to phosphatidylglycerol has a considerable effect on the thermal transition of the protein [45]. All curves shown in Fig. 1 are reproducible following repated heating and cooling, even after brief exposure to temperatures as high as 70 °C. Similar experiments with dipalmitoylphosphatidylcholine indicated that there is no binding and no effect on the transition of the neutral phospholipid even in the presence of high concentrations of ribonuclease (30 mg in 1.5 ml containing 3 μmol of dipalmitoylphosphatidylcholine.

Similar results to the above were obtained when ribonuclease was mixed with pre-formed sonicated phosphatidylglycerol vesicles, and a typical curve is shown in Fig. 4 (curve e). In this case, the vesicles were made at 45 °C, and then equilibrated at 24 °C, at which point they were mixed with ribonuclease in 10 mM NaCl, pH 7.4. After incubation for 1 h at 24 °C, the white floculant, which formed immediately following the addition of the protein, was centrifuged (10 000 × *g* for 10 min) and the pellet examined in the calorimeter. Sonicated vesicles alone do not sediment at all under these conditions. During the first heating, a peak was obtained with a mid-point at 41 °C. The sample was then heated at 45 °C for 10 min, cooled and heated again. The tracing obtained during the second heating was superimposable to the one obtained earlier, with a second shallow peak centering at 56 °C.

The results described above, indicate that ribonuclease binds strongly to phosphatidylglycerol vesicles and can form precipitable material containing a high ratio of protein to lipid. However, the presence of the protein has no significant effect on the phase transition mid-point temperature (T_c) and a small but significant increase in the enthalpy (ΔH) of the transition. As will be discussed later, this is consistent with earlier results [46, 47] indicating that ribonuclease has only a minimal effect on the permeability of phospholipid vesicles and does not induce expansion of the area in phospholipid monolayers at the air-water interface at pressures above 24 dynes/cm^2. The overall conclusion that could be drawn from these results is that ribonuclease (which is positively charged at pH 7.4) adsorbs at the lipid-water interface and binds to negative charges without penetrating into the bilayer interior, and thus it does not interfere with the packing of the acyl chains.

The results obtained when phosphatidylglycerol was suspended in 10 mM NaCl buffer at pH 7.4 in the presence of increasing amounts of polylysine is shown in

Fig. 2. The addition of 0.2 mg polylysine to 3 μmol (2.3 mg) of dipalmitoylphosphatidylglycerol produces (curve b) two peaks one of which (41 °C) is similar to that obtained with pure phosphatidylglycerol and a second at higher (46.5 °C) temperature. Addition of 0.8 mg of polylysine in a similar system (curve c) produces only one peak at 48 °C. Higher amounts of polylysine (4 mg) produce a main peak at 44.5 °C with a minor at 48 °C (curve d), although the minor peak does not appear during reheating (broken line). The enthalpy of transition (ΔH) was found to be increased from 8 kcal/mol for the control dipalmitoylphosphatidylglycerol in 10 mM NaCl, to 9, 11 and 12 kcal/mol in the presence of 0.2, 0.8 and 4.0 mg polylysine, respectively. Where the double peak was present, the ΔH was calculated from the area under both peaks.

Although the results with polylysine are complicated by the appearance of more than one new peak, it is clear that its presence induces a 3 to 8 C increase in the T_c of phosphatidylglycerol, and also a substantial increase in the ΔH of transition. These data can be interpreted as a stabilization of the bilayer, possibly due to the neutralization of the negative charges of dipalmitoylphosphatidylglycerol. A similar increase in T_c for dipalmitoylphosphatidylglycerol was reported earlier in the presence of 3 mM Mg^{2+} [10, 41] and dimyristoylphosphatidic acid in the presence of low concentrations of both Ca^{2+} and Mg^{2+} [48]. Similarly, the basic myelin protein has been reported to stabilize preparations of dilaurylphosphatidylglycerol [24]. Stabilization of bilayers by polylysine is compatible with a localization at the lipid-water interphase, bound primarily by ionic interactions to the phosphate groups. Earlier studies have shown that the addition of polylysine to phosphatidylserine vesicles induces only a small increase in Na^+ efflux rate [46], and that the polypeptide undergoes a conformational transition from random coil to α-helix following binding to the vesicles [49]. It is possible that the double peak (Fig. 2 curve b) might be related to different conformational states, or to two phases, one unreacted phosphatidylglycerol and another (higher melting) phosphatidylglycerol-polylysine complex.

Interactions with basic myelin protein, cytochrome c etc. (group 2)

Addition of increasing amounts of the basic myelin protein (A1 protein) to phosphatidylglycerol during suspension in 10 mM NaCl at pH 7.4 induces a large decrease in the T_c of this phospholipid (Fig. 3). Under similar conditions, A1 protein has no effect on the thermotropic properties of dipalmitoylphosphatidylcholine. As can be seen in Fig. 3, addition of 0.5 mg of the A1 protein in the suspending buffer (curve b) produces membranes containing 10 % protein (calculated by dry weight) with a small but definite effect on the thermotropic behavior. Thus, the main endothermic peak of phosphatidylglycerol is unaltered except for slight broadening, but the pre-melt has disappeared. The presence of 2 mg A1 protein gives membranes containing 51 % protein by weight. In this case (curve c) the main endothermic peak is shifted to 40.2 from 41.2 °C with a shoulder at approx. 37 °C. When similar amounts of lipid and A1 protein were incubated at 100 mM NaCl (instead of 10 mM NaCl), the main peak was again shifted by 1 °C to lower temperatures with a second peak at 34 °C (tracing with broken line in Fig. 3). The presence of 4 mg of A1 protein (at 10 mM NaCl, curve d) also produced two peaks at 39 and 33 °C, and the sample contained 65 % protein by weight. Higher amounts of A1 protein in the original suspending buffer (8 mg) produce membranes with only one peak appearing at 31.2 °C,

containing 73 % protein by weight (curve e).

The enthalpy of transition was decreased in the presence of A1 protein, from 7.8 kcal/mol for pure dipalmitoylphosphatidylglycerol, to 7.5 kcal/mol for membranes containing 10 % protein, to 5.1 kcal/mol for membranes with 51 % protein, to 4.2 kcal/mol for those with 65 % protein and 5.5 kcal/mol when 73 % protein was present (curve e). Where more than one peak was present the sum of the area under both peaks was used to calculate ΔH. All the tracings shown in Fig. 3 are reproducible upon second heating, and no separate protein denaturation peak was obtained at

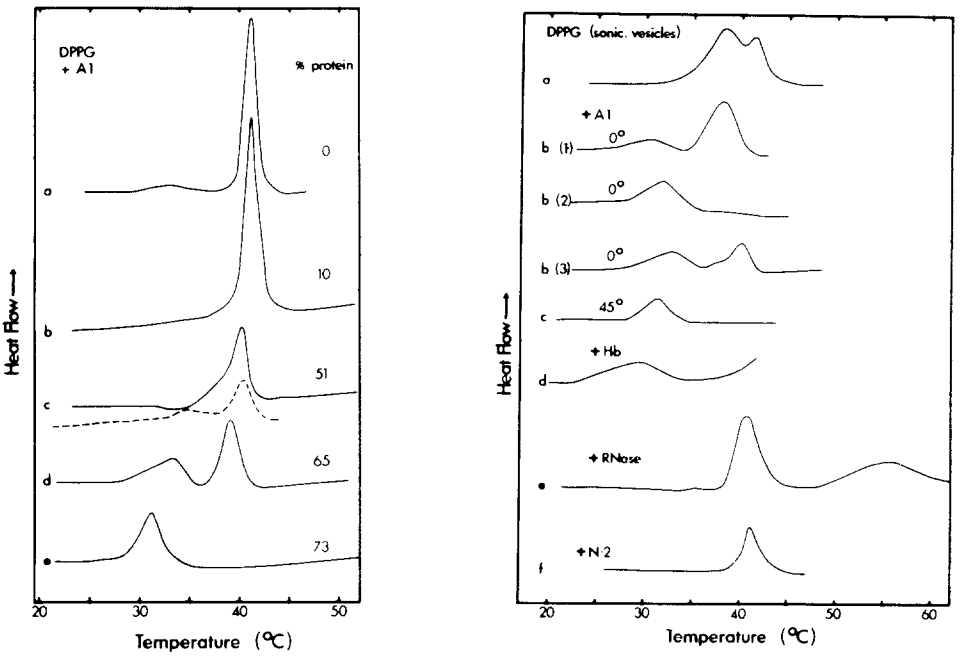

Fig. 3. Differential scanning calorimetry of dipalmitoylphosphatidylglycerol (DPPG) membranes prepared in the presence of basic myelin protein (A1). Each mixture contained 3 μmol dipalmitoylphosphatidylglycerol in 1.5 ml of 10 mM NaCl buffer at pH 7.4 plus different amounts of A1 protein: a, none; b, 0.5 mg; c, 2 mg; d, 4 mg; and e, 8 mg. Each pellet obtained from each mixture contained: a, 1.5 μmol phosphate; b, 1.0 μmol phosphate, 0.1 mg protein; c, 0.76 μmol phosphate, 0.62 mg protein; d, 1.31 μmol phosphate, 1.82 mg protein; and e, 0.72 μmol phosphate, 1.52 mg protein.

Fig. 4. Differential scanning calorimetry of sonicated vesicles of dipalmitoylphosphatidylglycerol (DPPG) before and after incubation with different proteins. Each mixture contained 3 μmol of dipalmitoylphosphatidylglycerol sonicated in 1.5 ml of 10 mM NaCl buffer, at pH 7.4. a, dipalmitoylphosphatidylglycerol vesicles after ultrafiltration for 3 h at room temperature and concentration to 0.2 volume; b, dipalmitoylphosphatidylglycerol vesicles were incubated with 5 mg A1 protein for 1 h at 0 °C. The precipitate was centrifuged (10 000 × g for 10 min at 24 °C) and transferred in the calorimeter pan at 0 °C: (1), curve obtained during the first heating of the b sample; (2), curve obtained from the same sample during second heating; (3), same sample except stored at 0 °C overnight; c, sample similar to b except the vesicles were originally incubated with A1 protein for 1 h at 45 °C; d, dipalmitoylphosphatidylglycerol vesicles incubated for 1 h at 24 °C with 20 mg hemoglobin; e, dipalmitoylphosphatidylglycerol vesicles incubated for 1 h at 24 °C with 20 mg ribonuclease; f, dipalmitoylphosphatidylglycerol vesicles incubated for 1 h at 24 °C with 1 mg N-2 apoprotein (water-soluble form).

higher temperatures.

The results discussed above indicate that when A1 protein is bound to acidic phospholipid membranes it can have a large effect by decreasing both the T_c and ΔH of the endothermic transition. The effect is very small when the membranes contain 10 % protein by weight but it becomes substantial in the region of 50–70 %. This effect can be interpreted as fluidization of the bilayer, induced by partial "penetration" of the protein into the hydrocarbon interior. The presence of two endothermic peaks (Fig. 3d) is a complicating factor and not easy to explain. Further studies are necessary in order to establish whether the low melting peak indicates the presence of lipid domains around A1 protein molecules or the formation of a completely separate phase.

In order to investigate the ability of A1 protein to interact with either "frozen" or "fluid" phosphatidylgylcerol bilayers we performed the experiment described in Fig. 4. In this case, pre-formed, sonicated vesicles in 10 mM NaCl, pH 7.4, were incubated with A1 protein at 0 °C and also at 45 °C. In both cases, the addition of A1 protein produced aggregation of the vesicles. The precipitate obtained at 0 °C gave a differential scanning calorimetry tracing shown in curve b(1), of Fig. 4. Two peaks are apparent, a minor one at 30 °C, and a major one at 38 °C. The minor peak corresponds to that obtained when dipalmitoylphosphatidylglycerol was suspended in the presence of 8 mg of A1 protein (Fig. 3, curve e). The major peak corresponds to the one obtained with sonicated vesicles alone* (Fig. 4, curve a). It is thus reasonable to conclude from curve b, that most of the phosphatidylglycerol present in the precipitate is unaffected by the presence of A1 protein. However when the same sample was reheated following the brief exposure to 42 °C and immediately cooled, curve b(2) was obtained indicating that now all phosphatidylglycerol melts at a lower temperature with considerably reduced enthalpy. Curve b(3) was also obtained with the same sample, after further incubation at 0 °C overnight. The presence of a substantial peak at 40 °C indicates that the effect of A1 protein is reversible. Curve c was obtained by an identical mixture to that in b, but which was incubated initially at 45 °C and gives a single low temperature peak.

The above results could be interpreted as follows: The low temperature endothermic peak obtained with phosphatidylglycerol when it was incubated with A1 protein at temperatures above the T_c, was due to partial "penetration into" and "deformation" of the bilayer. Such interaction would probably involve non-polar (hydrophobic?) associations but it is dependent on initial electrostatic interactions. The non-electrostatic or hydrophobic interaction was inhibited when the bilayer was frozen (below the T_c). It occurred only after brief exposure to temperatures above the T_c, and was at least partially reversed following incubation at temperatures below the T_c. In this later case, the situation could be characterized as a freezing-out of the protein.

The interactions of two other proteins, cytochrome *c* and hemoglobin, with phosphatidylglycerol showed similar effects and are described in Fig. 5. The top curve

* The sonication of dipalmitoylphosphatidylglycerol dispersions produces considerable broadening of the endothermic peak [41] and a shift of the mid-point from 41 to 38 °C. The shoulder at 41 °C shown in Fig. 4 (curve a) could be due to either the presence of residual large vesicles which have not been affected by sonication, or to a re-appearance of the original 41 °C peak during the time period of ultrafiltration. This latter phenomenon has been observed before [41] following incubation of sonicated phosphatidylglycerol vesicles for 20 h at 45 °C.

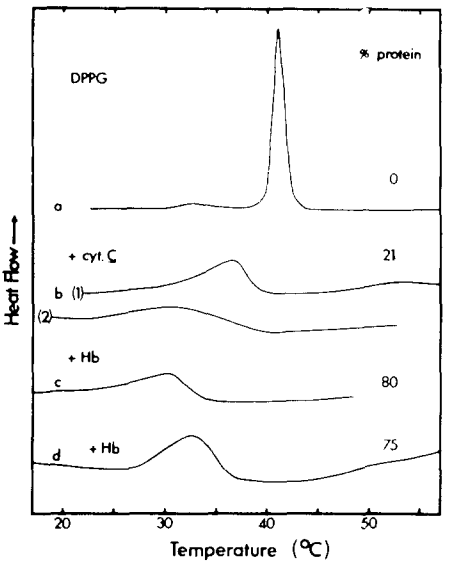

Fig. 5. Differential scanning calorimetry of dipalmitoylphosphatidylglycerol (DPPG) membranes prepared in the presence of cytochrome *c* and hemoglobin. Each mixture contained 3 μmol dipalmitoylphosphatidylglycerol in 1.5 ml of 10 mM NaCl (except for d, 100 mM NaCl) buffer at pH 7.4 containing different amounts of protein: a, dipalmitoylphosphatidylglycerol alone; b, dipalmitoylphosphatidylglycerol plus 10 mg cytochrome *c*.The pellet contained 1.0 μmol phosphate and 0.2 mg protein: (1), first heating curve; (2), third consecutive heating curve; c, dipalmitoylphosphatidylglycerol plus 25 mg hemoglobin; pellet contained 0.96 μmol phosphate and 3.16 mg protein; d, same as c except in 100 mM NaCl.

was obtained with pure phosphatidylglycerol dispersed in 10 mM NaCl buffer at pH 7.4. The two middle curves (b) were obtained in the presence of 10 mg of cytochrome *c* in the buffer during dispersion. The pellet obtained from this mixture contained 21 % protein by weight. Curve b(1) was obtained during the first heating scan, and curve (2) during second scan, following brief exposure to temperatures up to 57 °C. It is evident that cytochrome *c* induces a decrease in the T_c (from 41 °C) to 36.5 °C during the first scan and to 30 °C during the second. A broad peak centering at 54 °C during the first heating scan is probably due to heat denaturation of the protein. The difference between the first and second scans could be the result of partial denaturation of cytochrome *c*. The enthalpy for the transition obtained from the first scan was 4.2 kcal/mol, a value considerably lower than that of pure phosphatidylglycerol. The lowering of the T_c of acidic phospholipids by cytochrome *c* has been reported before [22, 23] and has been interpreted as due to the electrostatic interactions between the lipid and the protein. We interpret this effect as the result of non-polar contacts of the protein with the bilayer, an explanation identical to that given earlier for the effects of A1 protein and consistent with previously proposed models [50, 51, 47].

The two lower curves shown in Fig. 5 were obtained by suspending phosphatidylglycerol in buffer containing 25 mg hemoglobin in 10 mM (curve c) and 100 mM (curve d) NaCl buffer, at pH 7.4. In both cases the T_c was lowered to 30 and 32.5 °C and the ΔH to 3.1 and 3.8 kcal/mol, respectively. The amount of protein present in the pellet was very large, 80 and 75 %, respectively, by weight. The overall effect is

thus similar to that obtained by cytochrome *c* and A1 protein. Hemoglobin has already been reported to increase drastically the permeability of phospholipid vesicles [52, 42]. In contrast to the other two proteins however, hemoglobin binds to, and increases the permeability of phosphatidylcholine vesicles. In contrast to cytochrome *c*, its interaction with acidic phospholipids is not inhibited by increasing the salt concentration from 10 to 100 mM. It is thus possible that the interaction of hemoglobin with phospholipid bilayers does not depend on initial electrostatic binding (at least at this pH 7.4, which is above the isolectric point) as seems to be the case for cytochrome *c*. Its interaction with neutral phosphatidylcholine membranes is similar to that observed with the proteolipid apoprotein, to be discussed below. The interaction of hemoglobin with pre-formed sonicated vesicles at 24 °C is shown in Fig. 4 (curve d), with results similar to the above.

All the interactions discussed in this section resulted in drastically reduced T_c and ΔH for the melting of phosphatidylglycerol membranes. The three proteins producing this effect, A1 protein, cytochrome *c* and hemoglobin, all expanded monolayers of acidic phospholipids at the air-water interface, and also produced large increases in the Na^+ efflux rate through acidic phospholipid vesicles. We interpret their effect as a fluidization of the bilayer due to "partial penetration" and consequent "deformation" of the packing of the phospholipid acyl chains.

Interactions of myelin proteolipid apoprotein (N-2 apoprotein) and gramicidin (group 3)

The major apoprotein from human myelin proteolipid [28] has been studied recently in reconstituted model systems. It has been shown to induce the appearance of intramembranous particles in freeze fractures of phospholipid vesicles [53]. Its effects on the thermotropic properties of dipalmitoylphosphatidylcholine are shown in Fig. 6. The tracing marked a on the upper left hand side was obtained with pure phosphatidylcholine dispersed in water. Identical results were obtained at 100 mM NaCl. The three tracings on the right hand side were obtained in the presence of increasing amounts of N-2 apoprotein, giving final precipitates containing 20 % (curve d), 32 % (curve e) and 52 % protein (curve f) by weight. The calculated ΔH for the main endothermic peak in each case was 6.3, 4.6 and 4.4 kcal/mol, respectively. In all three cases the T_c of the main peak remains essentially unaltered (within 1 °C) although it becomes broader at high protein-to-lipid ratios, and the pre-melt is eliminated in samples containing more than 20 % protein.

Similar results were obtained irrespective of whether the initial mixing of the N-2 apoprotein with the lipid was accomplished in chloroform/methanol, water or buffer [54]. Furthermore, the results were similar with both phosphatidylcholine and phosphatidylglycerol. As reported elsewhere [54] it appears that the ΔH for the lipid transition decreases linearly as the percentage of the protein increases up to approx. 50 % by weight. Such samples containing equal weights of lipid and protein were centrifuged in sucrose density gradients where they settled on the top of a cushion of 40 % sucrose (density between 1.08 and 1.17 g/ml) clearly separating from pure lipids and protein alone [54]. It appears then that the N-2 apoprotein can bind to a variety of phospholipids and the resulting bilayers containing up to 50 % protein by weight still exhibit an endothermic transition at the same temperature but of reduced enthalpy (ΔH).

These results were rather unexpected in view of the earlier observations indi-

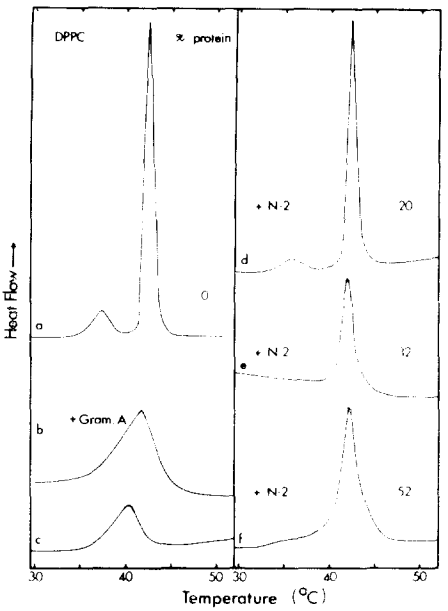

Fig. 6. Differential scanning calorimetry of dipalmitoylphosphatidylcholine (DPPC) prepared in the presence of gramicidin A (gram. A) and proteolipid apoprotein (N-2). a, dipalmitoylphosphatidylcholine alone, 3 μmol suspended in 1.5 ml of 10 mM NaCl buffer, pH 6.5; b, 6 μmol dipalmitoylphosphatidylcholine were mixed with 1 mol gramicidin A in chloroform/ethanol (1 : 1, v v) evaporated to dryness, and suspended in 0.1 ml of 100 mM NaCl buffer at pH 7.4. Of this mixture, 10 μl were used as sample; c, same as b, except the lipid was dipalmitoylphosphatidylglycerol; d, 3 μmol dipalmitoylphsosphatidylcholine were mixed in chloroform/methanol/water with 0.72 mg of N-2 apoprotein. Evaporated to dryness and suspended for 1 h at 42 °C in 1 ml of 100 mM NaCl, pH 7.4; pellet contained 1.12 μmol phosphate and 0.24 mg protein; e, same as above except 1.1 mg of N-2 apoprotein added initially; pellet contained 1.1 μmol phosphate, and 0.42 mg protein; f, Same as in d, except 2.5 mg of N-2 apoprotein added initially. The mixture was suspended in water and finally layered on a discontinuous sucrose density gradient. After centrifugation for 6 h at 100 000 · g the material recovered from the interface between 20 and 40 % sucrose was resuspended in 1 ml water, centrifuged at 10 000 · g for 10 min, and the pellet used as sample. It contained 1.22 μmol phosphate and 1.1 mg protein.

cating that the N-2 apoprotein can increase the permeability of phospholipid vesicles and expand the area of phospholipid monolayers at constant pressure of 24 dynes cm^2 [42]. Furthermore, the lipophilicity of this protein and its ability to induce the appearance of intramembranous particles in freeze-fractures of phospholipid vesicles [53], both suggested that the N-2 apoprotein can penetrate deeply into the bilayer. We interpret the present calorimetric results as indicating that the N-2 apoprotein, although embedded in the bilayer, does not perturb the acyl chain packing of the bulk lipid (no decrease in T_c). The phospholipid molecules around the embedded protein could be more strongly bound to it, or perhaps relatively immobilized by the protein [26] and consequently do not participate in the cooperative melting of the bulk lipid (decrease in ΔH).

In order to substantiate the above conclusions we studied the interaction of another hydrophobic peptide, gramicidin, with the same lipids. The results are shown in curves b and c of Fig. 6. The initial molar ratio of phospholipid to gramicidin in

these samples was 6 to 1. The thermograms obtained with both lipids indicate a considerable broadening of the main endothermic peak, substantial lowering of the ΔH (to approx. 5 kcal/mol) but only a small effect on the mid-point (T_c) of the main endothermic peak (lowering by 1 °C). Previous studies by several investigators have shown that gramicidin A forms ion-conducting channels composed of a dimer that spans the length of the bilayer [55, 56]. Such channels probably have a conformation favoring the localization of the methylene groups in the perimetry of a helix, thus maximizing non-polar contacts with the interior of the bilayer [55]. It is apparent from the results obtained here with gramicidin, that the presence of this molecule does not affect drastically the mid-point for the transition of the bulk lipid, although the transition becomes considerably broader, with a lower ΔH. A recent study [23] reported that gramicidin A produces a considerably more pronounced lowering of the mid-point of the dipalmitoylphosphatidylcholine transition (4 °C). It is not clear at present what the difference between the two results is due to. However, we note that the mid-point for the main dipalmitoylphosphatidylcholine transition is also at variance between the two studies (42. 5 °C here and 46 °C in ref. 23).

Correlation of calorimetric studies with effects on vesicle permeability and monolayer area expansion

An examination of the effects of the same proteins on other properties of phospholipid membranes reveals some relevant relationships. Fig. 7 compares the ability of five proteins and polypeptides to increase the permeability of sonicated phosphatidylserine vesicles to Na^+. The proteins are added outside the vesicles in 10 mM NaCl buffer at different concentrations and the efflux of $^{22}Na^+$ from inside the vesicles is taken as an index of its self-diffusion rate. It can be seen clearly that the A1 protein and N-2 apoprotein both induce large increases in Na^+ efflux (approx. 1000-fold) at low concentrations (approx. 1 mg/ml). Cytochrome *c* induces a similarly large increase but at much higher concentration (10–40 mg/ml). However, neither polylysine, nor ribonuclease induce comparably large increases, even at high concentrations (10–30 mg/ml).

The ability to increase the permeability of phospholipid vesicles is not an exclusive property of membrane proteins, since lysozyme [46], hemoglobin [52, 42] and even albumin [57] can induce similar large increases. Unfolding of ribonuclease has been shown to augment its ability to increase the permeability of vesicles, and the film pressure of monolayers [47]. It has been proposed [47] that the ability of proteins to increase the permeability of phospholipid membranes is based on their ability to either "penetrate" or "deform" the acyl chain packing within the bilayers [47]. This proposal was based on a correlation of increased permeability in vesicles, with increased film pressure ($\Delta\pi$) in monolayers induced by several proteins.

We have recently extended these studies by measuring the ability of proteins to increase the area of phospholipid films kept at constant pressure. Table I gives the results obtained with several of the proteins discussed earlier. It can be seen that ribonuclease has no effect at all at 24 dynes/cm^2 while cytochrome *c*, A1 protein and N-2 apoprotein, all have large effects and produce considerable expansion in film area. Such increase in area per molecule could be rationalized by either a "penetration" or "deformation" model. However, the important result is that ribonuclease produces no expansion, which correlates well with its minimal effect on phosphatidylserine

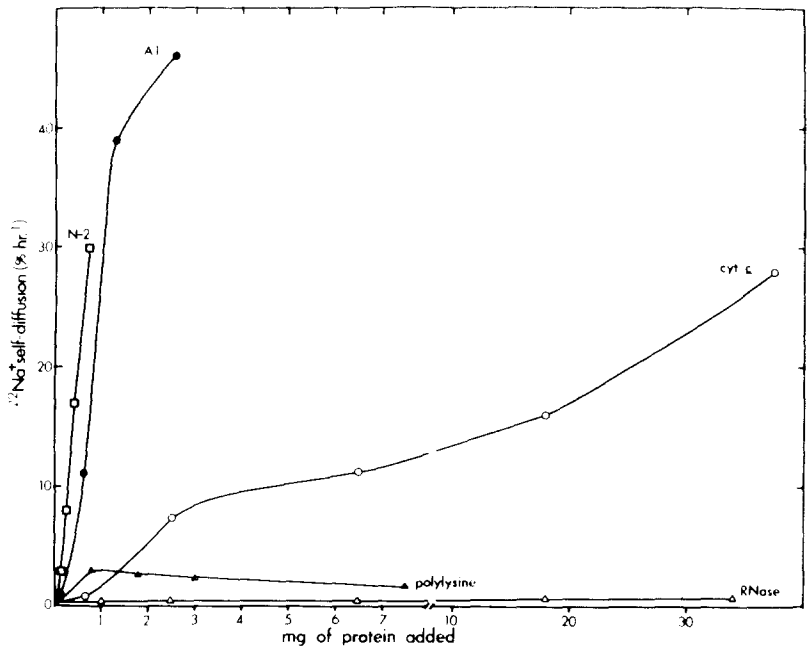

Fig. 7. Effect of different proteins on permeability of phospholipid vesicles to Na^+. Each mixture contained 1 μmol of phosphatidyl-serine vesicles sonicated in 100 mM NaCl, pH 7.4, in a final volume of 1 ml. Proteins were dialyzed against the same buffer and were added outside the preformed vesicles, at the indicated amounts. Permeability was measured by following the efflux of $^{22}Na^+$ initially trapped within the interior of the vesicles. The mixtures were dialyzed at 24 °C against 10 ml of same buffer, which was changed at 1-h intervals. □ □, major apoprotein from human myelin proteolipid (N-2); ○ ○, basic myelin protein (A1); ● ●, cytochrome *c*; ▲ ▲, poly-L-lysine; △ △, ribonuclease.

TABLE I

AREA EXPANSION OF PHOSPHOLIPID MONOLAYERS AT CONSTANT SURFACE PRESSURE INDUCED BY PROTEINS

The monolayer area expansion was measured with a phosphatidylserine film at an initial film pressure of 25 dynes/cm^2, at 25 °C, in 10 mM NaCl bulk phase, pH 7.4. The protein was injected under the film and the bulk phase stirred with magnetic stirrer. The barrier was moved to increase the area in order to compensate for any increase in film pressure (1 dyne/cm^2 [42]. The values of increase in area were obtained 1 h after injection of the protein. They represent averages of two experiments which were reproducible within 10 % of the figure given.

Protein	Concentration (μg/ml)	Increase in area (%)
Ribonuclease	2.5	0
Cytochrome *c*	2.0	38
Basic myelin protein (A1)	1.6	65
Proteolipid apoprotein (N-2)	1.2	60

vesicle permeability, and on the T_c of phosphatidylglycerol membranes. On the contrary, all the other proteins which expand the monolayers, also increase vesicle permeability. It is tempting to speculate that proteins which tend to "deform" the bilayer acyl chain packing by inducing non-polar contacts close to the lipid-water interface, would have a large "fluidizing" effect (i.e. A1 protein and cytochrome *c*). On the other hand, proteins or peptides which can assume conformations that allow for extensive non-polar interactions with the lipid and can thus penetrate into the bilayer interior either half way or fully, might have a minimal effect on acyl chain packing.

GENERAL DISCUSSION AND CONCLUSIONS

A summary of the effects of different proteins on the properties of phospholipid bilayers and monolayers is given in Table II and a schematic, idealized, representation of the three types of interactions is shown in Fig. 8. Interaction 1 (simple adsorption), is represented by ribonuclease and polylysine, interaction 2 (adsorption and deformation) by cytochrome *c* and A1 protein and interactions 3a and 3b (penetration) by N-2 apoprotein and gramicidin A. It is clear that combinations of different types of interactions might also occur with different proteins. Type 1 interaction which involves simple adsorption at the interface could be induced either by electrostatic interactions as shown here and proposed originally by Danielli and Davson [58], or by specific interactions involving non-charged polar groups, such as carbohydrate residues [59]. Type 2 interaction, which involves fluidization of the bilayer, would tend to decrease membrane thickness as shown in Fig. 8. The degree of non-polar association between protein and lipid would depend on the fluidity of the lipid, as has been shown here by the reversibility of the A1 protein effect and also by the inhibition

TABLE II

SUMMARY OF EFFECTS OF DIFFERENT PROTEINS ON SEVERAL PROPERTIES OF PHOSPHOLIPID BILAYERS AND MONOLAYERS

Details of the interactions and quantitative aspects are given in detail in the text.

Group	Protein	(*A*) Area per molecule in monolayers	(*P*) Vesicle permeability to sodium	(ΔH) Enthalpy of acyl chain melting	(T_c) Temperature of acyl chain melting
1	Ribonuclease	None	small increase	increase	none
	Polylysine	—	small increase	increase	increase
2	Basic myelin protein (A1)	Increase	large increase	decrease	decrease
	Cytochrome *c*	Increase	large increase	decrease	decrease
3	Major myelin proteolipid (N-2)	Increase	large increase	decrease	none
	Gramicidin A	Increase	large increase	decrease	none

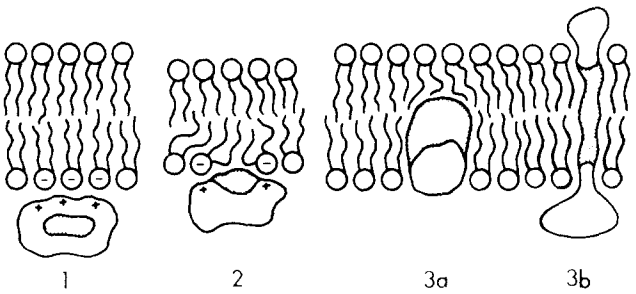

Fig. 8. Schematic representation of the three types of lipid protein interactions as explained in the text.

of protein penetration by cholesterol, reported earlier [42]. Type 3 interaction is similar to that proposed by Singer and Nicolson [11] and also by others [60, 61] for the localization of intrinsic proteins within the lipid bilayer. The extensive hydrophobic contacts with the lipid could be achieved either by the presence of a hydrophobic peptide segment [62, 63] which becomes the anchoring point or by oligomeric arrangements that shield the more polar part of the protein monomer. Evidence indicating inhibition of molecular motion of the acyl chains adjacent to such protein-lipid interface has been reported [26]. Such interaction would not be specifically detected by the calorimetric method used here, except indirectly by the decrease in ΔH, which could be interpreted as indicating that fewer phospholipid molecules participate in the cooperative melting of the bulk lipid.

Another pertinent point that can be made from the calorimetric data is the relative effect of the different proteins on the size of the cooperative unit for the melting of the phospholipid acyl chains. The size of the cooperative unit can be calculated from the ratio of the enthalpies derived from van't Hoff equation to the corresponding calorimetric enthalpies, and involves the assumption of a two state process [45, 64]. Within the limitations of the above calculations, we have determined that the cooperative unit of phosphatidylglycerol bilayers is reduced by approx. 58 % (from 58 to 24 molecules) in the case of cytochrome *c* (Fig. 5, curve b (I)), but only by 6 % (from 84 to 79) in the case of N-2 apoprotein and phosphatidylcholine (Fig. 6, curve e) and 26 % (from 58 to 43) in the case of ribonuclease (Fig. 1, curve b). The comparatively large effect of cytochrome *c* in reducing the size of the cooperative unit can be interpreted as loss of long-range order within the phospholipid bilayer, and is compatible with the "deformation" model discussed above. On the other hand, the smaller effects of N-2 apoprotein and ribonuclease on the size of the cooperative unit, indicate that these proteins do not interfere greatly with the long-range order of the phospholipid bilayer. This is supportive again of the interactions type 1 and 3 discussed above. In the former case (ribonuclease) the protein interacts strictly with the polar headgroups, without penetrating into the interior. In the latter case (N-2 apoprotein) the protein is at least partly embedded into the bilayer but it interacts with only a limited number of phospholipid molecules without perturbing the rest of the bilayer.

The implicit assumption in all the above considerations is that the overall lipid bilayer structure is preserved during the interactions with the different proteins. However, a change in the transition temperature for the melting of the acyl chains induced

by the presence of a particular protein could be interpreted also as due to the formation of an entirely new phase structure, such as hexagonal, not necessarily involving lipid lamellae. Parallel X-ray diffraction studies would be ideally suited to this problem, but have not been applied consistently yet to the various interactions discussed here. The only well-studied case is that of cytochrome *c* interaction with various acidic lipids [36, 50, 67], which shows the presence of a lamellar phase involving both lipid and protein layers. Of the other proteins, the interaction of A1 with brain lipids [68] and the interaction of N-2 apoprotein with egg lecithin (Rand, P., private communications) both indicate the presence of well-ordered lipid lamellae.

The lipid-protein interactions studied in this paper were not undertaken for their strict relevance to specific membrane functions, but as well-defined examples relevant to the general question of how different proteins affect the properties of lipid bilayers [19, 20, 22]. The data indicate that while some electrostatic interactions result in stabilization of the bilayer, others induce considerable fluidization. Most importantly, it appears that lipid-protein complexes that involve largely non-polar (hydrophobic) associations have no effect on the bulk-lipid T_c, although they reduce proportionately the heat of transition. In any case, the studies reported here indicate the complexity of the problem of interpretation of thermotropic phase transitions in biological membranes [19, 20, 22]. In addition to the diverse effects discussed above, any assignment of phase changes in a multicomponent system has to take into consideration the recently reported role of divalent metals in inducing both a large increase in the T_c or acidic lipids [10, 48, 24, 41] and also their separation into domains [65, 66].

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