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INTERACTIONS OF PROTEINS AND CHOLESTEROL WITH LIPIDS IN BILAYER MEMBRANES

WOLFGANG KLEEMANN* and HARDEN M McCONNELL**

Stauffer Laboratory for Physical Chemistry, Stanford, Calif 94305 (U S A)

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SUMMARY

Mixtures of lipids and protein, the ATPase from rabbit sarcoplasmic reticulum, were studied by freeze-fracture electron microscopy and by measurement of the amount of fluid lipid with the spin label 2,2,6,6-tetramethylpiperidine-1-oxyl (TEMPO) In dimyristoyl phosphatidylcholine vesicles the protein molecules were randomly distributed above the transition temperature, T_t, of the lipid and aggregated below T_t For mixtures of dimyristoyl and dipalmitoyl phosphatidylcholine the existence of fluid and solid domains was shown in the temperature interval predicted from earlier TEMPO measurements When protein was incorporated into this lipid mixture, freeze-fracture particles were randomly distributed in fluid lipids, or aggregated when only solid lipids were present

In mixtures of dimyristoyl phosphatidylcholine with cholesterol the protein was distributed randomly above the transition temperature of the phosphatidylcholine Below that transition temperature the protein was excluded from a banded phase of solid lipid in the case of 10 mol % cholesterol In mixtures containing 20 mol % cholesterol, protein molecules formed linear arrays, 50–200 nm in length, around smooth patches of lipid

Phase diagrams for lipid/cholesterol and lipid/protein systems are proposed which account for many of the available data A model for increasing solidification of lipid around protein molecules or cholesterol above the transition temperature of the lipid is discussed

INTRODUCTION

It is by now well known that lipid molecules in lipid bilayers undergo phase transitions and phase separations [1, 2] At temperatures below the phase transition

Abbreviation TEMPO, 2,2,6,6-tetramethylpiperidine-1-oxyl, HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid

* Present address Max-Planck-Institut fur biophysikalische Chemie, 34 Gottingen-Nikolausberg, G F R

** To whom correspondence should be addressed

the lipid hydrocarbon chains are relatively rigid and extended, above the phase transition they are flexible and have a higher probability of *gauche* conformations The influence which protein or cholesterol molecules have on the phases, phase transitions and phase separations is not understood quite as well Neither is the influence of the lipids on the distribution of protein molecules in the membranes

From a number of studies [3–10], it is known that the distribution of particles which are believed to be manifestations of protein molecules, in a membrane fracture face changes with temperature and lipid composition The protein molecules appear to be excluded from patches of solid lipid The composition of these membranes is, however, not defined as much as one would like it to be Studies were therefore undertaken on reconstituted bilayers made from single lipids or simple mixtures and a defined protein The particular protein in these studies was the $(Mg^{2+}+Ca^{2+})$-ATPase from rabbit sarcoplasmic reticulum and it was incorporated into dimyristoyl phosphatidylcholine or mixtures of dimyristoyl and dipalmitoyl phosphatidylcholine or dimyristoyl phosphatidylcholine and cholesterol Evidence is also presented for the existence of a solid and a fluid phase in bilayers formed from equimolar mixtures of dimyristoyl and dipalmitoyl phosphatidylcholine at temperatures predicted from the phase diagram of Shimshick and McConnell [2]

METHODS

Preparation of lipid-exchanged ATPase

The $(Mg^{2+}+Ca^{2+})$-ATPase was isolated by minor modifications of the procedure of Warren et al [11, 12] Sarcoplasmic reticulum vesicles in 250 mM sucrose in gradient buffer (1 M KCl, 50 mM Tris Cl, pH 8, 2 5 mM dithiothreitol) and a 10 % solution of sodium cholate (Sigma) were mixed at a ratio of 0 5 mg cholic acid/mg sarcoplasmic reticulum protein A pellet of undissolved material was removed by centrifugation at 160 000 $\times g$ for 25 min at 4 °C The supernatant was layered on top of a 60–20 % (w/v) continuous sucrose gradient in gradient buffer and centrifuged at 90 000 $\times g$ for 24 h at 4 °C An opaque band formed at approx 45–50 % (w/v) sucrose and was collected through the bottom of the tube The 1 ml fractions were assayed for protein, lipid, ATPase activity and by gel electrophoresis The purified ATPase contained small amounts of protein of higher molecular weight The lipid content was 16 % (w/w) and ATPase activity at 37 °C was 7 3 units/mg protein These values correspond closely to those reported by Warren et al [11]

The dimyristoyl and dipalmitoyl phosphatidylcholines (Calbiochem) used for lipid exchange and reconstitution were pure by thin-layer chromatography, but gas chromatography indicated the presence of small amounts of shorter fatty acids Dry dimyristoyl phosphatidylcholine was dissolved in cholate solution, using 1 mg cholate/mg phospholipid Dissolution was aided by brief sonication in a bath sonicator (Heath Systems) Gradient buffer and ATPase protein solution were added to give a total volume of 2 0 ml with a protein lipid ratio of between 1 1 and 1 2 (w/w) After incubation for approx 1 h at room temperature or several hours in the refrigerator, the samples were layered atop step gradients comprised of 0 6 ml 50 % (w/v) and 1 9 ml 20 % (w/v) sucrose in gradient buffer The gradients were centrifuged at 160 000 $\times g$ for 15 h at 25 °C and an opaque band was removed with a pipet from the top part of the 50 % (w/v) sucrose layer It was homogenized and assayed The

lipid substitution was repeated using a protein lipid ratio of between 1 1 and 1 3 (w/w) The band of doubly lipid-exchanged ATPase was homogenized assayed and used in dialysis-reconstitution experiments

Lipid exchange had to be carried out at 25 C, otherwise most of the activity was lost Similar adjustments were recently found necessary by Warren et al [13] The doubly lipid-exchanged ATPase had activities of the order of 3 units/mg

Formation of vesicles

Lipids were dissolved in chloroform and mixed in the appropriate amounts. The solvent was evaporated by passing a stream of nitrogen into the tube and last traces of solvent were removed in a vacuum desiccator overnight To the dried lipids was added 0 5 ml dialysis buffer (8 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES), pH 7 25 with KOH, 250 mM sucrose, 400 mM KCl, 1 5 mM $MgSO_4$, 0 1 mM $CaCl_2$, 1 mM EDTA, 1 mM NaN_3 [14]) Liposomes were formed by vortexing with intermittant heating to above the transition temperature of the higher melting lipid The liposomes were then broken up by brief sonication in a bath sonicator and cholate was added at a level of 1 mg detergent/mg lipid plus protein The solution cleared upon shaking, except when the cholesterol content exceeded 20 mol % of the total lipid Protein was added to give a lipid protein ratio of 2 1 or 9 1 (w/w) After incubation for several hours at 4 C and warming up to the desired dialysis temperature, generally 30 C, the samples were dialysed against 150–300 vols of dialysis buffer Dialysis was continued for 2–4 days with buffer changes approximately every 12 h For the final 6–12 h the nonionic polymeric adsorbent Amberlite XAD-2 (Mallinckrodt) was included in the dialysis buffer at a level of 10 g/l, to sequester any residual detergent [11, 12] The dialysate was centrifuged at 29 000 × *g* for 20 min and the supernatant pipetted off The pellet was dispersed by slightly heating and vortexing

Reconstitution, here meaning the incorporation of protein into bilayer vesicles of synthetic lipid, led to ATPase activities of about 1–2 units/mg Ca^{2+} uptake was not generally measured and was quite low in most cases Not all protein incorporated into bilayers, but formed some sort of aggregates with lipid, especially when the specific activity was very low or when dialysis was carried out at low temperatures

Freeze-fracturing

Small sample droplets of dispersed pellets from tubes equilibrated at the desired temperature were pipetted onto copper planchets resting on a metal block of the same temperature The planchets were equilibrated for another minute in a chamber of the metal block and then rapidly plunged into partially solidified Freon 22 (du Pont) Samples were kept under liquid N_2 until fracturing in a Balzers BAF301 Freeze-Etching Device Fracturing was carried out at −116 °C with no etching Replicas were floated off on water, kept on sodium hypochlorite solution (Chlorox) for 1 h, rinsed on water and transferred to ethanol They were picked up on bare 400 mesh copper grids directly from ethanol or after stretching on water Micrographs were taken on 35 mm film with an initial magnification of about 12 000 × in Philips EM200 electron microscopes All prints are at a final magnification of 45 000 and are mounted with the shadow coming approximately from the bottom

Analytical techniques

Protein was estimated by the method of Lowry et al [15], using a solution of bovine serum albumin (Mann Research Laboratories) as standard Phospholipid content was determined by assaying for inorganic phosphate after digestion of the sample with perchloric acid [16] Enzymatic activity of the ATPase (ATP hydrolysis) was determined using the coupled enzyme assay described by Warren et al [11] A unit of activity is that amount of enzyme which produces a turnover of 1 μmol substrate in 1 min Polyacrylamide gel electrophoresis was carried out in the presence of sodium dodecyl sulfate according to the method of Weber and Osborn [17] Fatty acid constituents of the lipid samples were analysed by gas chromatography of the methyl esters

EPR measurements of the TEMPO spectral parameter f were carried out as described by Shimshick and McConnell [2] TEMPO (2,2,6,6-tetramethylpiperidine-1-oxyl) is a small spin label that dissolves in water and in fluid hydrophobic bilayer regions The spectral parameter f is a measure of the amount of fluid lipid present and can be used to detect the gel to liquid-crystalline (solid to fluid) phase transition in lipid bilayers and membranes

RESULTS

Spin label measurements

The TEMPO spectral parameter f was measured for samples of dimyristoyl phosphatidylcholine with and without protein and for an equimolar mixture of dimyristoyl and dipalmitoyl phosphatidylcholine with protein (Fig 1) The solid to fluid transition was rather sharp, centered at 23 °C, for dimyristoyl phosphatidylcholine As increasing amounts of protein were included, the transition broadened considerably The low temperature end did not change or decreased slightly in tem-

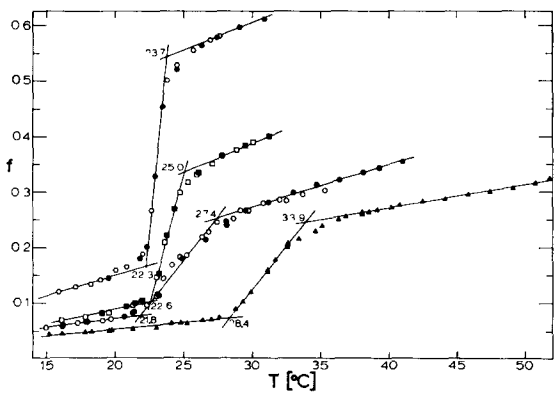


Fig 1 TEMPO binding curves for reconstituted vesicles Open symbols are points obtained by cooling the sample, closed symbols are points obtained during subsequent heating (◆) Dimyristoyl phosphatidylcholine, (■) 90 % dimyristoyl phosphatidylcholine with 10 % (w/w) protein, (●) 70 % dimyristoyl phosphatidylcholine with 30 % (w/w) protein, (▲) 75 % lipid (equimolar mixture of dimyristoyl and dipalmitoyl phosphatidylcholine) with 25 % (w/w) protein

perature, the upper end shifted to increasingly higher temperatures and the change in df/dT was less sudden

For the mixture of dimyristoyl and dipalmitoyl phosphatidylcholine the influence of the protein on the transition is far less pronounced An equimolar mixture of these lipids exists as solid and fluid phases in equilibrium at temperatures between 29 and 34 °C [2] The high-temperature end of the phase separation region is hardly changed at all by inclusion of the protein The slightly lower temperature for the beginning of melting could be due partly to the fact that this sample contained a little more than an equimolar amount of dimyristoyl phosphatidylcholine, as judged from gas chromatograms

The samples were vesicles tightly packed by centrifugation in a clinical centrifuge The relative amounts of water and lip d are therefore probably not the same Thus it cannot be concluded that the reduction in the absolute value of f at temperatures above the transition was due to a permanent solidification of a fraction of the lipid by the protein, although this remains a possibility as will be discussed later

Freeze-fracture studies

The ATPase was incorporated into vesicles made from dimyristoyl phosphatidylcholine, a mixture of dimyristoyl and dipalmitoyl phosphatidylcholine and mixtures of dimyristoyl phosphatidylcholine with cholesterol In pure dimyristoyl phosphatidylcholine the particles in the fracture face, which are manifestations of protein molecules, were distributed randomly when a sample was quenched from above T_t (Fig 2a) Any ordering that can be seen is likely to be due to processes taking place during quenching of the sample In particular, it has been shown that the pattern of disordered ridges or jumbled lines was absent when higher quenching speeds were employed [18] At temperatures below T_t the protein molecules were excluded from large banded regions These bands are characteristic for saturated phosphatidylcholines below the transition temperature [19] and thus the freezing lipid must have excluded the protein from the crystal Similar observations on dark-

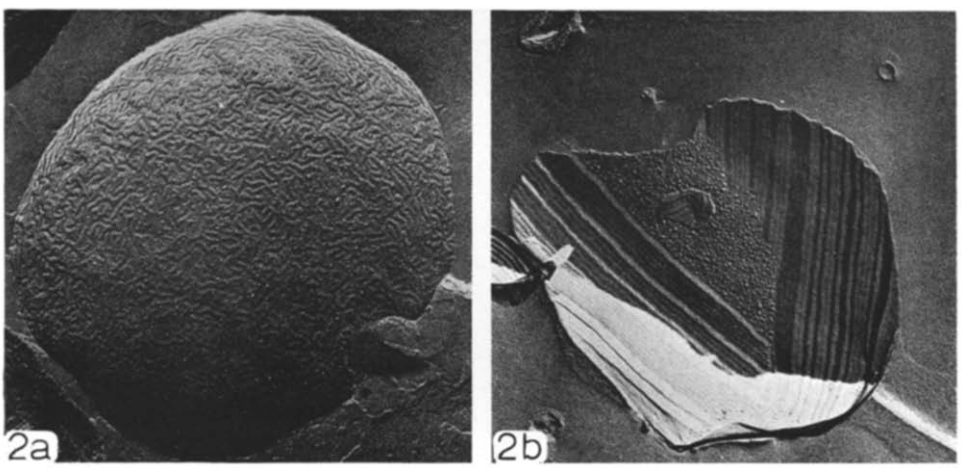


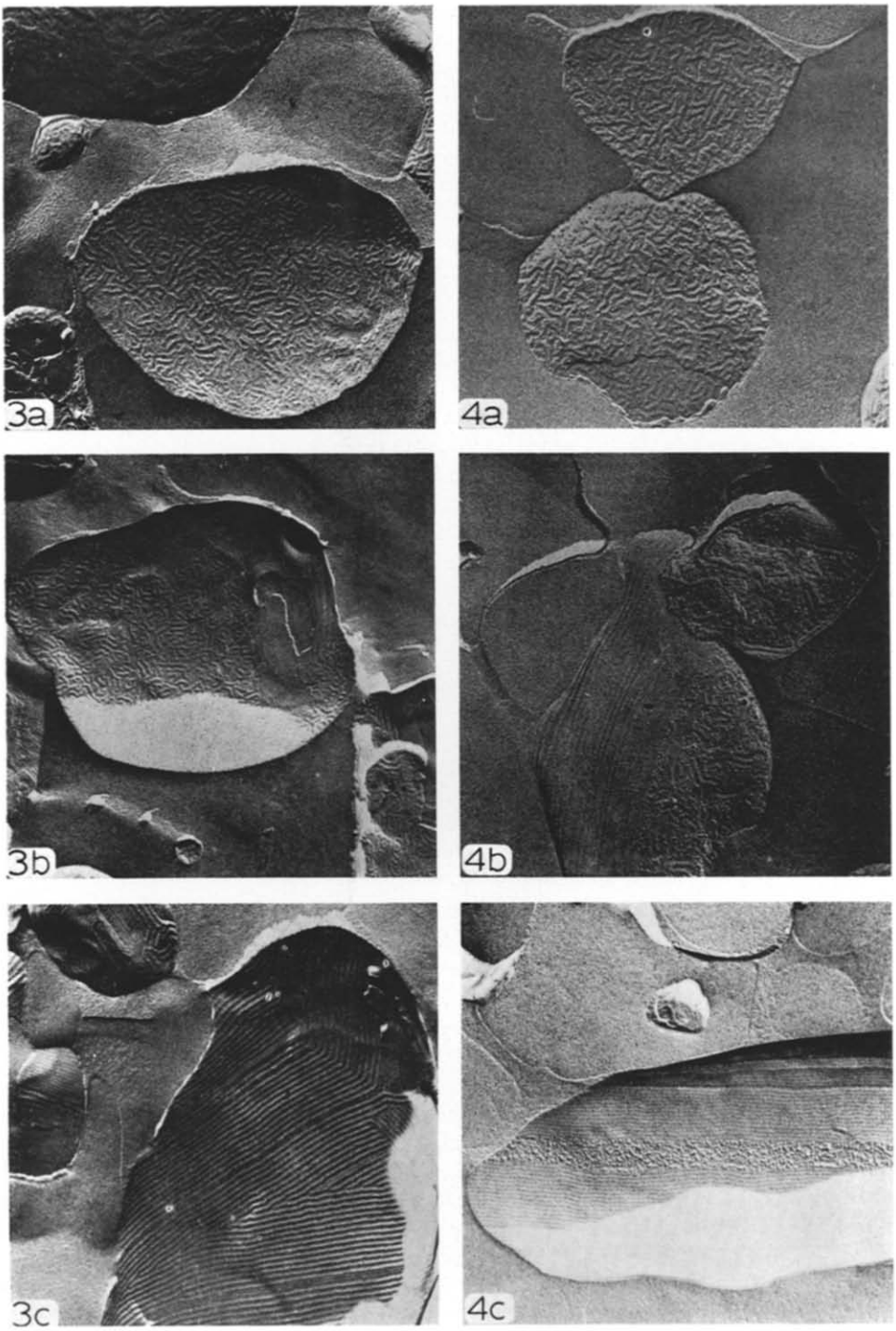
Fig 2 Freeze-fracture micrographs of vesicles made from dimyristoyl phosphatidylcholine with 10 weight % protein Samples were quenched from (a) 30 C and (b) 13 C

adapted rhodopsin were reported by Chen and Hubbell [20] However, bleached rhodopsin did not form a separate phase under the same conditions, but stayed randomly distributed Glycophorin, the major glycoprotein from the human red blood cell [21], was also dispersed when it was included in a pure lipid, e g dimyristoyl or dipalmitoyl phosphatidylcholine, and examined below the transition temperature of the lipid [22] It thus appears that for the ATPase or dark-adapted rhodopsin the favorable crystal packing energy of the pure lipid outweighs the entropy which would be gained by random mixing For bleached rhodopsin and glycophorin this is not the case, or the energy difference is so small that only extremely low cooling rates would induce the formation of two phases, one of pure lipid and another with protein and only very little lipid

In pure phosphatidylcholines the transition from the gel to the liquid-crystalline state occurs over a very narrow temperature interval, about 1 or 2 °C wide as judged from EPR measurements [2] For binary mixtures of phosphatidylcholines there exists an extended temperature region (fluid and solid region) in which solid and fluid lipid patches coexist, as was inferred from differential scanning calorimetry [1] and from spin label data [2] Mixtures in which the two lipids differ by more than two carbon atoms in the acyl chains generally show partial solid phase immiscibility, also called monotectic behavior [1] For these mixtures, freeze-fracture studies have revealed the presence of two kinds of domain between the two transition temperatures and, in the case when one lipid was dioleoyl phosphatidylcholine, also below the lower transition temperature [19] Another mixture, that of dipalmitoyl and dielaidoyl phosphatidylcholine, was carefully examined in freeze-fracture studies by Grant et al [23] The predictions from a spin-label-derived phase diagram were all verified, in that solid and fluid lipid phases coexisted in the fluid and solid region of the phase diagram in approximately the expected proportions This mixture is believed to form solid solutions over the entire range of compositions [24], but the region of fluid and solid coexistence is rather large, from 13 to 32 °C No differential scanning calorimetry curves have been reported on this mixture, and so it is not known whether this system exhibits two peaks like monotectic mixtures, or only one broad one like mixed crystals

In Fig 3 freeze-fracture electron micrographs are shown for an equimolar mixture of dimyristoyl and dipalmitoyl phosphatidylcholine This system forms solid solutions at all compositions as judged by the criteria of spin labeling [2] or differential scanning calorimetry As only one peak is observed in differential scanning calorimetry, this mixture is said to cocrystallize [25], and the possibility of observing two different phases in coexistence was therefore not investigated [19] From the spin-label-derived phase diagram, the coexistence of solid and fluid patches was predicted between 29 and 34 °C, in an equimolar mixture [2] In these experiments, as expected, only very little fluid lipid was found at 31 °C, whereas at 33 °C the fluid phase made up at least half of the lipid areas exposed by the fracturing process (Fig 3b) At temperatures above T_f all lipid had a "jumbled" appearance (Fig 3a) and below T_s no jumbled or smooth lipid areas were observed, but only parallel ridges (Fig 3c)

When the ATPase was incorporated into equimolar mixtures of dimyristoyl and dipalmitoyl phosphatidylcholine, the appearance of the lipid was not changed The protein particles were randomly distributed in the fluid phase (Figs 4a and 4b) as long as fluid phase was present When only solid lipid was present, the protein formed a



Figs 3 and 4 See opposite page for legends

separate phase with little lipid (Fig 4c) and thus areas densely covered with particles and other areas with the ripple pattern were seen in the freeze-fracture micrographs

In experiments on an equimolar mixture of dielaidoyl and dipalmitoyl phosphatidylcholine the ATPase was also excluded from the solid phase lipids at all temperatures and randomly distributed in the fluid phase above T_f This behavior is analogous to that reported for glycophorin by Grant and McConnell [26] When only solid lipid was present, the ATPase again formed a separate phase

Mixtures containing cholesterol

The effect of cholesterol on phase transitions of phosphatidylcholines has been studied by a number of methods, such as differential scanning calorimetry [27, 28], X-ray diffraction [29], nuclear magnetic resonance [30] and electron paramagnetic resonance spectroscopy [31] The picture that emerges from these studies, summarized in the review article by Oldfield and Chapman [32], is that cholesterol somehow fluidizes a lipid that is below its transition temperature, but rigidifies a lipid above T_t Cholesterol is said to create an "intermediate fluid condition" In differential scanning calorimetry measurements the heat of the transition disappears at about 33 mol% cholesterol [27] and the sharp 4 15 Å X-ray diffraction line disappears at about the same composition [29] A specific 2 1 interaction (mol phosphatidylcholine mol cholesterol) was therefore postulated and a phase separation into regions of 2 1 complex and pure phosphatidylcholine Recent calorimetric evidence [28, 33] suggests that cholesterol at concentrations up to 20 mol % shows a preference for the species with the lower transition temperature in equimolar phosphatidylcholine mixtures which show phase separation below T_s The phase formed by cholesterol and the lower melting lipid thus would contain 33 mol % cholesterol At higher cholesterol concentrations the heat of the upper transition is also decreased Phillips and Finer [34] favor a definite 1 1 molecular complex, based primarily on NMR studies and the observation by Bourgès et al [35] that the maximum amount of cholesterol incorporated into phosphatidylcholine is 50 mol % in the presence of excess water. However, Lecuyer and Dervichian [36] noted that the properties of this system did change at a mol ratio of 2 1

In freeze-fracture preparations cholesterol diminished the intensity of the bands observed for pure dimyristoyl phosphatidylcholine below T_t at concentrations of less than 20 mol % At higher cholesterol concentrations no bands were observed, but bands of low amplitude could escape detection [5, 25] At all temperatures all areas looked homogeneous, i e either banded or smooth Thus, if a phase separation does occur, the domains do not differ in appearance, or the domain size is very small, for instance, bands of different composition could exist below T_t of the phosphatidylcholine

From the studies of the pure and mixed phosphatidylcholine systems it was known that the ATPase preferred a fluid environment Thus there existed the possi-

Fig 3 Freeze-fracture micrographs of vesicles made from an equimolar mixture of dimyristoyl and dipalmitoyl phosphatidylcholine Samples were quenched from (a) 39 °C, (b) 33 °C and (c) 23 C

Fig 4 Freeze-fracture micrographs of vesicles made from an equimolar mixture of dimyristoyl and dipalmitoyl phosphatidylcholine with 25 weight % protein Samples were quenched from (a) 37 °C, (b) 32 °C and (c) 20 °C

bility that the protein molecules could serve as a marker for the fluid phase, if fluid and solid phases should coexist above the transition temperature of the phosphatidylcholine The results of freeze-fracture experiments on preparations of dimyristoyl phosphatidylcholine containing 10 or 20 mol % cholesterol and 10 or 33 weight % protein are shown in Figs 5 and 6 Two phases, one protein-rich and another protein-poor, were observed below T_t (at 15 C) for samples containing 10 mol % cholesterol (Fig 5a) The protein-poor phase had the band pattern which is observed in the absence of protein The amount of this phase depended on the amount of protein present, being much larger for preparations with only 10 weight % protein A reversible decrease in the amount of protein-poor phase occurred when the sample was heated towards 23 °C, the transition temperature of dimyristoyl phosphatidylcholine An intermediate temperature point is shown in Fig 5b In samples with only 10 weight % protein the decrease of protein-poor phase was not as easily detected because only little protein-rich phase was present initially Above 23 °C protein particles were distributed rather randomly over the entire vesicle face (Figs 5c and 5d) When only 10 weight % protein was present indications of small disordered lines (or bands) were seen in the lipid (Fig 5d), but these are thought to be due to rearrangements taking place during quenching of the sample for freeze-fracture

Samples containing 20 mol % cholesterol and 33 weight % protein showed a qualitatively different behavior below the transition temperature (T_t) of dimyristoyl phosphatidylcholine The fracture faces showed large areas completely free of

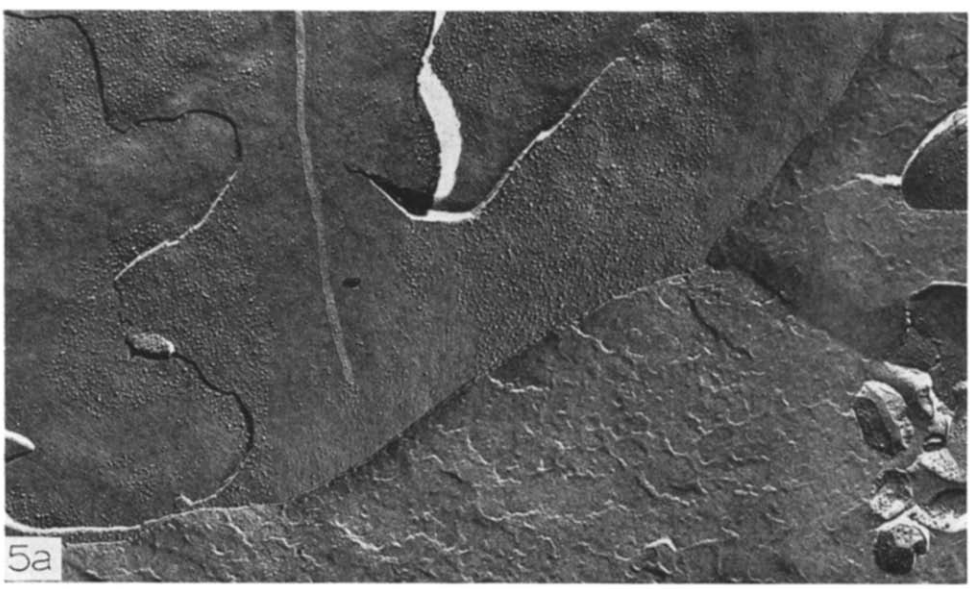
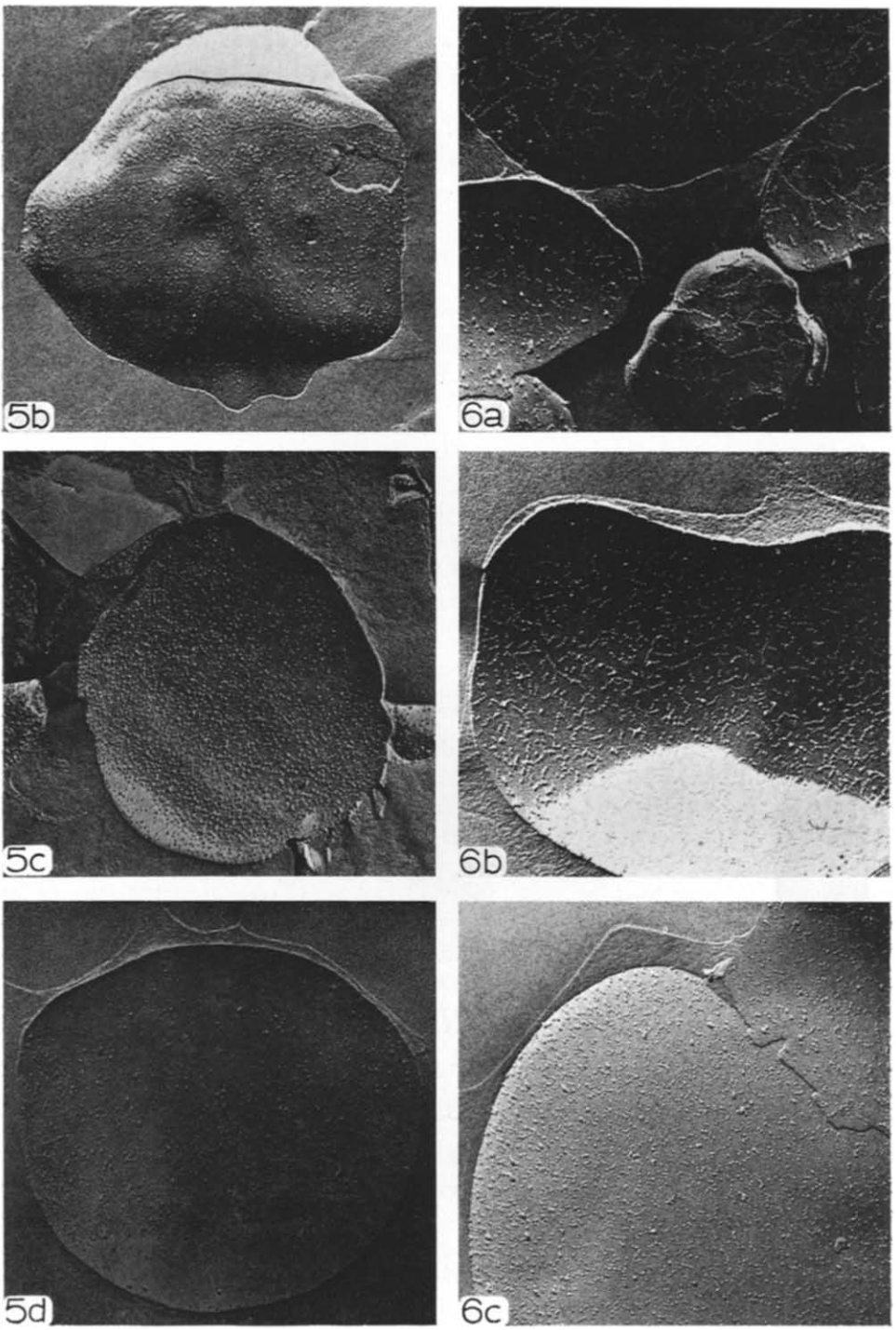


Fig 5 Freeze-fracture micrographs of vesicles made from 90 mol % dimyristoyl phosphatidylcholine and 10 % mol cholesterol with (a–c) 33 weight % and (d) 10 weight % protein Samples were quenched from (a) 15 C, (b) 19 5 C, (c) 31 C and (d) 25 C

Fig 6 Freeze-fracture micrographs of vesicles made from 80 mol % dimyristoyl phosphatidylcholine and 20 mol % cholesterol with 33 weight % protein Samples were quenched from (a) 13 C, (b) 16 5 C and (c) 23 5 C



Figs 5 and 6 See opposite page for legends

particles in a number of cases (top of Fig 6a), but most vesicles were covered with a network of protein molecules The particles formed linear arrays of 50–200 nm in length, and these lines joined at angles, rather than round bends Each line was only one particle wide and sets of lines partially or totally enclosed patches that were smooth and contained no particles The size of the patches decreased reversibly as the temperature was raised towards 23 °C, thereby indicating a rather high lateral mobility at these temperatures below the transition of dimyristoyl phosphatidylcholine, much higher than is found for cholesterol-free samples (Fig 6b) Samples quenched from above 23 °C showed a small amount of order (Fig 6c), which could be due to insufficient quenching speeds, like the pattern observed in unsaturated fatty acid auxotrophs of *Escherichia coli* when the cells were quenched without fixing from a temperature above the onset of lateral phase separation [8] The increase in size of the particle-free patches below 23 °C indicates an increasing amount of long range order This order appears to be quite different, however, from the one observed for 10 mol % cholesterol preparations, as the domains are much smaller and have no apparent linear structure in them

Experiments on mixtures of 80 mol % dimyristoyl phosphatidylcholine and 20 mol % cholesterol with 10 weight % protein gave very inhomogeneous vesicle populations Some vesicles and liposomes had no protein at all, others behaved like preparations with 33 weight % protein or 10 mol % cholesterol The inhomogeneity may have been due to incomplete solubilization by cholate

DISCUSSION

Binary systems of phosphatidylcholines have been successfully described by phase diagrams, derived from spin label data [2, 24] and verified by freeze-fracture observations [22, 23] Shimshick and McConnell [37] derived a phase diagram for mixtures of dimyristoyl phosphatidylcholine with cholesterol from measurements of the TEMPO spectral parameter From that phase diagram it could be concluded that, from 0 to 20 mol % cholesterol, two solid phases coexist below 23 C (T_s, the temperature corresponding to a point on the "solidus"), the transition temperature of dimyristoyl phosphatidylcholine For larger cholesterol concentrations a solid solution would form which had increasingly higher melting points (T_s) At temperatures above T_s, but below T_f (on the "fluidus") solid and fluid domains should coexist

Freeze-fracture studies on mixtures with or without protein gave no indications for the presence of extended domains of different fluidity above T_t They did, however, suggest a change in the properties of the system at 20 mol % cholesterol Another indication for a change at this composition was provided by the solubility behavior of lipid/cholesterol mixtures in cholate solution Mixtures with more than 20 mol % cholesterol did not give a clear solution and therefore could not be used in protein reconstitution experiments Other protein-detergent combinations may provide additional insight [38] Other evidence relating to this system, especially to the 2 1 and 1 1 complexes, has already been mentioned

At present it seems almost impossible to account for all the available data in one phase diagram Comparison between the behavior of protein molecules and cholesterol molecules may prove helpful and hypothetical phase diagrams (Fig 7) will aid the discussion even though proof for them is far from complete At cholesterol

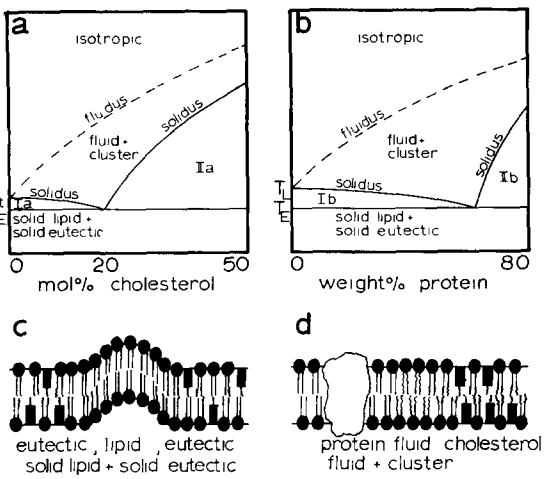


Fig 7 Hypothetical phase diagrams for (a) lipid/cholesterol and (b) lipid/protein mixtures The "fluidus" and the "solidus" would be derived from spin label data, they do not correspond to the thermodynamic equivalent T_t is the transition temperature of the pure lipid (dimyristoyl phosphatidylcholine), T_L is the temperature or temperature region at which the combined lipids form a protein-free solid and T_E is the eutectic temperature In the fluid+cluster phase lipid molecules around protein molecules or lipd-cholesterol complexes are rigidified This phase, which is essentially fluid, is in equilibrium with solid pure lipid in region I, or with solid complex in region II In (c) a possible structure of alternating bands of pure lipid and the eutectic is shown to explain the banded pattern observed in mixtures of 90 mol % dimyristoyl phosphatidylcholine and 10 mol % cholesterol The left half of (d) shows the rigidifying effect of protein molecules, the right half that of lipid-cholesterol complexes on near lipid molecules in the fluid+cluster phase

concentrations higher than 50 mol % a separate phase of crystalline cholesterol exists, thus the diagram valid for the bilayer phase terminates at that composition Likewise, a minimal amount of lipid must be associated with the protein in order to form bilayers The estimate of 20 weight % lipid seems reasonable in view of the numbers obtained by Jost et al [39] and Warren et al [11]

In measurements of the TEMPO spectral parameter the transition is broadened when cholesterol [37] or protein (Fig 1) are included with the pure phosphatidylcholine The lower end of the transition hardly changes, but the upper end is shifted to increasingly higher temperatures As of yet not many temperature points between the lower and the upper end of a phosphatidylcholine-protein system have been investigated, but the available data indicate that no separation into large domains of different composition and fluidity occurs Thus an increasing immobilization of the lipid molecules around a protein molecule, extending farther into the lipid phase as the lipid transition temperature is approached, may account for the decrease in TEMPO solubility In the Appendix a model for this solidification process is discussed, showing how a "break" in df/dT may occur and account for the T_f values In the case of cholesterol a cluster of 1 1 complexes may take the place of a protein molecule (Fig 7d) In neither case would large domains of different appearance show up in micrographs

As the temperature is decreased further to below the transition temperature of the lipid (T_L in Fig 7b), solid lipid forms which excludes the protein from the lipid

crystal Eventually the eutectic line (T_E) is passed and a eutectic of protein-lipid complex and extra lipid solidifies All systems investigated here have protein contents of less than the eutectic composition The above statements are in accord with the freeze-fracture data In lipid/cholesterol mixtures without protein no extended domains are seen [25], but if the phase diagram treatment is applicable to systems containing cholesterol, domains of different composition and fluidity must be present at certain temperatures Data from differential scanning calorimetry and X-ray measurements indicate some sort of transition up to 33 mol % or maybe higher at the transition temperature of the phosphatidylcholine or a little below, supporting the eutectic line in Fig 7a A possibility for compositional heterogeneity is indicated in Fig 7c, in which strips of pure phosphatidylcholine are shown to alternate with strips of eutectic The phosphatidylcholine is responsible for the ridges, and as the amount of pure phosphatidylcholine phase decreases between 0 and 20 mol % cholesterol, the ridges disappear over this composition range [25] The temperature interval between T_t and T_E is probably very small At cholesterol concentrations above 20 mol % a complex of cholesterol and phosphatidylcholine first solidifies As the whole system is very rich in cholesterol, TEMPO solubility is generally low (see Shimshick and McConnell [37]) and the lower temperature "break" detected is for the freezing out of the complex

As protein is incorporated into phosphatidylcholine/cholesterol mixtures, the lipid behaves as one component with the transition at T_L, approximately 23 °C T_E is approximately 16 °C In the case of 10 mol % cholesterol the phosphatidylcholine induces a long-range linearity, leading to large domain sizes and the banded pattern in the solid lipid phase The eutectic, at 20 mol % cholesterol, has domains growing from a center, with linear borders but without the long-range linearity The arrays of protein particles occupy the boundaries between domains of solid phosphatidylcholine-cholesterol eutectic The amount of lipid associated with the protein changes reversibly between 16 and 23 °C, as indicated by the phase diagram (Fig 7b) Therefore the domains of solid lipid change size between these two temperatures also

A 1 1 complex of phosphatidylcholine with cholesterol has been employed, as cooperativity effects may account for the phenomena at 33 mol % cholesterol [34] There is no doubt that some of the above speculations will be proven wrong, but the picture outlined here may stimulate new experiments and other ideas A relatively large amount of data on the interaction between lipids and protein molecules or cholesterol has been included in a unified picture

APPENDIX

Phase separations and the temperature dependence of spin label spectra

In the present paper specific examples, as well as references to earlier work, were cited, in which the temperature dependence of spin label (TEMPO) binding to lipid bilayer membranes, as well as to biological membranes, could be used to determine the temperatures corresponding to the onset (T_f) and completion (T_s) of lateral phase separations within the plane of these membranes For several cases these separations have been verified by freeze-fracture electron microscopy However, other examples were given in the present paper, where a strong temperature dependence of TEMPO binding was not correlated with lateral phase separations observable

in freeze-fracture preparations, as mentioned in the Discussion The purpose of this Appendix is to present a simple semiquantitative calculation that illustrates how certain interactions between membrane components can give rise to a strong temperature dependence of TEMPO binding, without the necessity of concomitant lateral phase separation

The essential physical idea is that, when a relatively rigid membrane component, such as cholesterol or a membrane protein, is inserted into a fluid phospholipid bilayer, it perturbs the phospholipid molecules in the immediate environment These perturbed molecules in turn perturb their neighbors, and so on, thus producing a "halo" around the relatively rigid membrane component (Fig 7d) Although this effect has been suggested by several authors [39, 40], it has apparently not been fully appreciated that the size of this halo may be quite large and highly temperature dependent For brevity, a very simple linear geometry is used in the following calculation Calculations more appropriate for membrane components will be given in subsequent work

Consider a bilayer membrane with a linear rigid barrier such that for $x \geqslant 0$ the membrane is composed of a single phospholipid species, e g dimyristoyl phosphatidylcholine, and for all $x < 0$ the membrane is rigid, i e. occupied by tightly packed protein molecules or lipids strongly immobilized by interaction with membrane proteins For $x \geqslant 0$, the strain energy of the lipid bilayer, per unit length, is approximated by the expression [41]

$$\tfrac{1}{2}k(n-\bar{n})^2+\eta\left(\frac{\mathrm{d}n}{\mathrm{d}x}\right)^2$$

Here k and η are (unknown) elastic constants of the bilayer, and n is a "structure parameter" For convenience n may be thought of as the average number of *gauche* bonds per lipid molecule, but other definitions may be possible or even preferable, as discussed later The term $\frac{1}{2}k(n-\bar{n})^2$ gives the elastic energy per unit length required to produce a uniform distortion in the bilayer, and $\eta(\mathrm{d}n/\mathrm{d}x)^2$ is the elastic energy per unit length required to produce a non-uniform distortion This second term corresponds to the tendency of neighboring molecules to be in the same physical state The quantity $\bar{n}$ is the average number of *gauche* bonds per molecule in the absence of the boundary at $x = 0$

The free energy of the system

$$F=\int_0^\infty\left[\tfrac{1}{2}k(n-\bar{n})^2+\eta\left(\frac{\mathrm{d}n}{\mathrm{d}x}\right)^2\right]\mathrm{d}x$$

is a minimum when n has the following dependence on x

$$n=\bar{n}(1-\exp\,(-x/\lambda))$$

where the correlation length λ is

$$\lambda=\sqrt{2\eta/k}$$

It is assumed that the lipids at the boundary, $x = 0$, are totally immobilized, so that $n(0) = 0$

Setting $n = n_t$ at the phase transition temperature of the pure lipid, T_t, and assuming a linear dependence of n on T above T_t,

$$\bar{n} = n_t + \mu(T - T_t)$$

is obtained

Since TEMPO binding drops precipitously when $T < T_t$, i e when $\bar{n} < n_t$, it is plausible to assume that all lipids are essentially frozen at points x where $n(x) < n_t$, and are fluid at points x where $n(x) > n_t$ Thus, the size of the rigid halo is x_t, which is the solution to the equation

$$n_t = \bar{n}(1 - \exp(-x_t/\lambda))$$

with a temperature dependence

$$x_t = \lambda \ln\left(1 - \frac{1}{\nu(T - T_t)}\right)$$

where $\nu = \mu/n_t$

In order to make a crude comparison of this result with TEMPO binding data, one can imagine that there are rigid boundaries of a lipid bilayer at both $x = 0$ and $x = 2X$ Thus, when $n(x)$ decreases with decreasing temperature to the point where $n(X) \leqslant n_t$, one may say that all the lipids are frozen At higher temperatures, the fraction of lipids in the fluid state, f' is

$$f' = 1 - \frac{x_t}{X} = 1 - \frac{\lambda}{X} \ln\left(1 - \frac{1}{\nu(T - T_t)}\right)$$

Fig 8 gives some illustrative plots of f' versus $(T - T_t)$ for various values of the two parameters, ν and λ/X These plots bear striking similarity to the observed binding of TEMPO to bilayer membranes containing cholesterol [37] or the ATPase, reported here Two particular features are to be noted Relative to TEMPO binding to the pure lipid for $T \geqslant T_t$ there can be a strong perturbation of TEMPO binding for

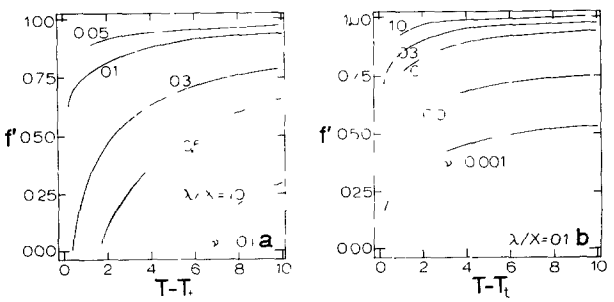


Fig 8 Calculated plots of the fraction, f', of the lipid in the fluid state The curves were obtained by calculating f' for values of $(T-T_t)$ from 0 to 10 C, in steps of 0 02 C Plots on the teletype were then traced In (a) the parameter $\nu = 0\ 1$ is held constant while λ/X is varied, in (b) ν is varied and $\lambda/X = 0\ 1$ is held constant Increasing values of λ/X can be looked at as a decrease in X, e g when the amount of protein is increased This leads (in a) to a broadening of the transition and a shift to higher temperature Decreasing values of $\nu(\mu)$ mean that, starting at n_t, a larger positive temperature change has to take place to reach a given value of $\bar{n}$, leading (in b) to a broadening of the transition

$T > T_t$ even though the low temperature break occurs at $T = T_t$ In other cases, the entire binding curve is displaced to higher temperatures The behavior clearly depends on whether or not the two "halos" overlap one another strongly

The above calculation demonstrates how a strong temperature dependence of TEMPO binding might arise through the ordering of lipid molecules around relatively rigid membrane components However, this calculation must be regarded as only schematic No proof has been given that our expression for the free energy density is adequate, and, most important, the proper choice of the "structure parameter" n is particularly critical Setting the structure parameter equal to the thickness of the lipid bilayer may well be a preferred definition In the case of a protein that spans the bilayer, such as glycophorin, the lipid-protein boundary condition would correspond to matching the bilayer thickness to the protein so that the hydrophobic regions overlap In this case, it is clear that a protein such as glycophorin might tend to increase or decrease the "fluidity" of the fluid bilayer state depending on whether the length of the hydrophobic stem of glycophorin was shorter or longer than the normal thickness of the hydrophobic interior of the bilayer Thus, the above theoretical calculations are uncertain, but can be refined and tested experimentally

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Drs S Mabrey and J M Sturtevant have kindly informed us that using a harshly sensitive scanning calorimeter they have been able to resolve the heat absorption in binary mixtures of dimyristoyl phosphatidylcholine and cholesterol into two components, one sharp and one broad The sharp component disappears at cholesterol concentrations above 20% Thus this concentration of cholesterol is now manifest in data obtained using spin labels, freeze-fracture electron microscopy and scanning calorimetry

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