

CRITICAL EFFECTS FROM LIPID-PROTEIN INTERACTION IN MEMBRANES

II. INTERPRETATION OF EXPERIMENTAL RESULTS

FRITZ JÄHNIG, *Max-Planck-Institut für Biologie, 74 Tübingen, Federal Republic of Germany*

ABSTRACT The effects arising from lipid-protein and lipid-cholesterol interaction are discussed within the framework of a general theoretical description presented in the preceding paper. Available experimental results are interpreted, and new experiments are proposed. In the fluid lipid phase proteins and cholesterol increase the lipid orientational order in their neighborhood, in the ordered phase they decrease it. This leads to a decrease of the latent heat at the ordered-fluid transition, which vanishes at a critical concentration of protein or cholesterol. Theoretical predictions for the critical concentrations agree with results from calorimetry. The approach to the critical point is accompanied by an increase of thermal fluctuations of the lipid order and an increase of the lipid response on small perturbations. Thus proteins and cholesterol increase the lipid specific heat, lateral compressibility, permeability, and lateral diffusion on both sides of the phase transition. Notions such as decrease of cooperativity or fluidity due to protein or cholesterol are reviewed in this context.

INTRODUCTION

In the preceding paper (1), I presented a theoretical study of the ordered-fluid transition in a lipid bilayer and its alteration by incorporated proteins or cholesterol. In the present paper, I compare the theoretical results with existing experimental data. The ordered-fluid transition was described within the framework of the Landau theory, a general theory for phase transitions, and the influence of proteins or cholesterol upon the lipids was incorporated into the theory by treating them as boundary conditions on the lipid order. The advantages of applying such a general theory are twofold: first, because of its universal validity one can profit from comparison with other phase transitions and, second, all effects occurring at the ordered fluid transition can be treated within a unifying framework. The correlation between static and dynamic behavior typical for phase transitions is especially important. An explanation will be presented as to why proteins or cholesterol make the lipids in the fluid phase more ordered on the one hand, and on the other hand, increase their permeability and lateral diffusion.

Experimental results on lipid-protein interaction have often been interpreted in terms of the presence of boundary lipid (2), or a decrease of cooperativity in lipid-lipid interaction and of membrane fluidity (3). I shall discuss these notions to give them a stronger physical basis.

THE ORDERED-FLUID TRANSITION IN PURE LIPID MEMBRANES

Static Lipid Order

The long-range order of lipid bilayer, which changes spontaneously at the ordered-fluid transition, was recognized in the previous article (1) as the orientational order of the lipid chains and was described by the orientational order parameter S . The ordered-fluid transition then turned out to be of first order, with a discontinuous change in S , in analogy to the nematic-isotropic transition in liquid crystals (4). In the fluid phase, S remains nonzero owing to the action of the surface pressure originating in the hydrophobic effect. The temperature dependence of S obtained is shown in Fig. 1.

Experimentally the order parameter S can be determined by deuterium magnetic resonance (DMR) (5), by electron spin resonance (ESR), and by fluorescence anisotropy (FA) (6, 7). In FA measurements S was found to behave qualitatively as in Fig. 1.

A remark on the notion of cooperativity is in order here. Cooperativity was introduced as a measure for the sharpness of, for instance, helix-coil transitions in polypeptides. The sharpness is determined by the number of amino acid residues, or, in general, by the number of particles interacting at the transition. In a lipid membrane (at least in the form of large liposomes) the interacting chains represent a many-particle system, which has practically infinite cooperativity and a sharp phase transition. The transition may, however, appear broadened in experiments that detect dynamic properties as discussed below.

Fluctuations and Response

A phase transition is often noticeable well before the actual transition when there is an increase of dynamic effects such as thermal fluctuations of the long-range order or the response to an external perturbation. Pretransitional¹ effects are typical for phase transitions of second order, e.g. the liquid-gas transition at the critical point. In the case of a first order transition, pretransitional effects also occur, although they are less pronounced. If the ordered-fluid transition at the temperature T^1 is approached from higher temperatures, the lipid system behaves as if approaching a second-order transition at T^1 , until the first-order transition is reached (Fig. 1). T^1 is the temperature that marks the end of metastable states below the phase transition. On approaching the phase transition from lower temperatures, the system behaves similarly. Therefore, the strength $\langle \delta S^2 \rangle$ of the order parameter fluctuations, their spatial extension measured by the coherence length ξ , and their life time or relaxation time τ show a pretransitional increase and become maximal at the ordered fluid transition, as illustrated in Fig. 2. The pretransitional increase is also exhibited by related response functions. An example is the specific heat, which increases because the enhanced fluctuations require energy. Another example is the lateral compressibility κ , which increases because of the coupling between lateral density and orientational order (1). Finally, if permeation and lateral diffusion to small particles in a membrane are assumed to proceed via lateral compression of the surrounding lipids, they also exhibit a pretransitional increase as in Fig. 2.

¹The expression "pretransitional" is in no way connected with the so-called pretransition found in some lipid membranes, but denotes the neighborhood of any phase transition, in our case the neighborhood of the ordered-fluid or main transition.

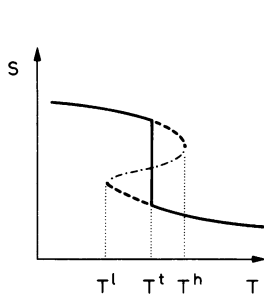


FIGURE 1

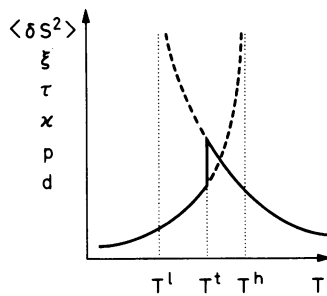


FIGURE 2

FIGURE 1 Variation of the orientational order at the ordered-fluid transition. The order parameter S changes abruptly at the transition temperature T^t . Metastable states (-) exist up to T^h and down to T^l , followed by unstable states (- -).

FIGURE 2 Pretransitional effects in the vicinity of the ordered-fluid transition. Increase of the magnitude of order parameter fluctuations $\langle \delta S^2 \rangle$, their coherence length ξ , their relaxation time τ , the lateral compressibility κ , the permeability p , and the lateral diffusion coefficient d .

Pretransitional fluctuations must be distinguished from co-existing ordered and fluid domains, which are often discussed in the interpretation of experimental results. Thermal order-parameter fluctuations vary smoothly in space to minimize their energy consumption and have a finite life time, whereas domains are visualized as stable regions with sharp boundaries. These domains demand much more than thermal energy and thus are less probable than thermal fluctuations (8), but are necessary to induce the first-order transition. Right at the transition temperature the system waits for a domain or nucleus that grows and then leads to the other phase. This makes the kinetics of the phase transition relatively slow (9).

Order parameter fluctuations should be detectable experimentally by light scattering and lead to an increase of the scattering intensity around the phase transition, as observed in the case of liquid crystals (4). This has not yet been observed with lipid membranes. However, fluctuations of the electric current through a lipid membrane were found to increase markedly at the phase transition (10). Although the coupling between electric current and orientational order has not been studied in detail, this result indicates a pretransitional increase of order parameter fluctuations.

The relaxation time of order parameter fluctuations is expected, from the analogy to nematic liquid crystals, to be of the order of 10^{-7} s (4). Such a relaxation time was indeed observed in dielectric relaxation measurements with lipid membranes and exhibits a maximum at the phase transition (11). Hence dielectric relaxation is sensitive to order parameter fluctuations. In time-resolved FA measurements another relaxation time ϕ in the range of 10^{-9} s is detected, which varies smoothly with temperature and shows a weak maximum at the phase transition (12, 13). The underlying relaxation process is the averaging over the fast fluctuations of chain orientation leading to the order parameter (1). This process is less dependent on cooperative lipid interaction than order parameter fluctuations, hence ϕ should be less affected by the phase transition. Frequently, the FA relaxation time has been interpreted in terms of the rotational motion of a rigid body in a viscous medium. In this case

ϕ is proportional to the so-called microviscosity of the membrane, which then also varies smoothly with temperature. This is expected for a viscosity at the high frequencies (10^9 Hz) relevant here. From measurements of deuterium spin-lattice relaxation a still shorter relaxation time of 10^{-10} to 10^{-11} s is obtained, which pertains to orientational fluctuations of the C—D bonds (5).

Proceeding to response functions, no increase of the specific heat has been detected by calorimetry. However, an increase of the lateral compressibility on both sides of the phase transition is observed in monolayer experiments (14). In aqueous dispersions, the lateral compressibility κ can be measured via the sound velocity $c_s \sim \kappa^{-1/2}$. Mitaku et al. (15) found that the sound velocity at 3 MHz exhibits a pronounced minimum at the phase transition, in agreement with the predicted maximum of the compressibility. The pretransitional decrease of c_s was extended over about 5°C on both sides of T^t . At T^t , a discontinuity occurred indicating a temperature dependence of κ as in Fig. 2. In mixtures of two lipids with different chain lengths, the discontinuity varied with the mixing ratio and even disappeared for a certain mixing ratio (16).

A maximum in the permeability of lipid membranes has been observed for some small particles such as sugars or metal ions (17). The same behavior was also found for a relatively large dye molecule (18). Here the permeation of the negatively charged amphiphilic dye is rate limited by the permeation of counterions. Thus the kinetics of the dye permeation reflects the permeation of small polar molecules. The temperature dependence of the lateral diffusion coefficient of lipid molecules at the phase transition is dominated by the abrupt decrease on passing from the fluid to the ordered phase. Whether there is a pretransitional increase in the fluid phase can not be inferred from existing experimental data (19).

A remark on the notion of membrane fluidity should be added. If fluidity is understood statically as a measure of structural disorder, then fluidity is high in the fluid phase and low in the ordered phase. If, on the other hand, fluidity is understood dynamically as a measure of structural lability, expressed e.g. by the permeability, then fluidity is maximal at the phase transition.

THE INFLUENCE OF PROTEINS OR CHOLESTEROL UPON THE ORDERED-FLUID TRANSITION

Static Lipid Order

A single protein or cholesterol molecule incorporated into a lipid membrane perturbs the lipid order (1). If the lipids are in the fluid state, the protein will act as a rigid body and increase the local orientational order parameter. Because of its uneven surface it may furthermore force the chains into a tilted direction, thereby altering the local preferred axis. In the ordered phase, the uneven surface of the protein again may impose a tilted orientation on neighboring lipid chains, in this case creating more space for fluctuations of the chains. Thus the local order parameter around the protein, if affected at all, is decreased. In the model studied in reference 1 I restricted myself to an alteration of the local order parameter, neglecting a change of the preferred orientation. Then the lipid-protein interaction is described by a boundary order parameter S_o at the protein surface, which is smaller than the unperturbed value S_u in the ordered phase and larger than S_u in the fluid phase. From the boundary

condition, the perturbation levels off in the surrounding lipid phase and $S(r)$ gradually attains the unperturbed value. This decay is smooth, since an abrupt change of the lipid order would require too much energy. As in the case of fluctuations of the order parameter, the decay is exponential with the coherence length ξ .

In this model of lipid-protein interaction, the protein influence on lipid order is not confined to the lipid chains in immediate contact with the protein, but extends further into the lipid phase because of the cooperativity of the lipid-lipid interaction. A measure of the radial extension is the coherence length, for which a theoretical estimate yielded $\xi \approx 15\text{\AA}$ at the phase transition. For a cylindrical protein with a radius of 10\AA , about 4 rings of lipid chains or 50 lipid molecules would be located within the coherence length. These lipids may be called "boundary lipids," although there is a continuous transition between perturbed and unperturbed lipids. Lipid molecules, which are tightly bound to the protein and no longer participate in the cooperative lipid order, would be regarded as part of the protein. This model is not restricted to lipid-protein interaction but is applicable to any rigid molecule in a lipid membrane, e.g. cholesterol.

The most direct experimental study of S_0 was done with a spin-labeled fatty acid, whose polar head was bound covalently to a protein so that the spin label sensed the immediate neighborhood of the protein (20). An immobilization of the label compared with pure lipid membranes was observed above the phase transition. This corresponds to a reduction of orientational chain fluctuations, hence an increase of the order parameter in the vicinity of the protein. The difference between the orders of boundary and unperturbed lipid can also be inferred from the quenching of intrinsic protein fluorescence by fluorophores in the lipid phase which are known to have a preference for either a fluid or an ordered lipid environment. Below T^t , the fluorophore with a preference for a fluid environment quenched protein fluorescence more strongly, indicating that the protein environment was more fluid than the unperturbed lipid (21). The analogous effect above T^t was less pronounced, because the other fluorophore had only a slight preference for an ordered environment.

Information about S_0 can also be obtained by detecting local order parameters throughout the membrane. The experimental technique used, however, must possess an intrinsic time for averaging over chain orientations which is short enough to detect local order parameters before the boundary lipids exchange with unperturbed lipids. ESR and FA accomplish this. In ESR experiments signals from individual rings of lipid chains around a protein could be resolved (22). Above T^t , the immobilization was found to decrease radially and to extend out to about six rings, in good agreement with our theoretical estimate based on the coherence length. In FA experiments the spatial average \bar{S} of $S(r)$ is measured (7). Above T^t , an increase of \bar{S} upon addition of protein or cholesterol was observed, indicating that S_0 was larger than the unperturbed order parameter (23, 24). Below T^t , \bar{S} decreased slightly implying that S_0 was smaller than the unperturbed value. Using another fast technique, namely Raman spectroscopy, an increase of the conformational chain order (more *trans* bonds) because of protein was detected above T^t , and again a decrease below T^t (25). Since the conformational order is part of the orientational order, this result parallels the one from FA. In DMR, on the other hand, the intrinsic time for averaging is longer than the time for exchange of lipid molecules between boundary and unperturbed regions. Hence DMR detects an order parameter which is the spatial average over the chain orientations in the whole

membrane. This order parameter was found to decrease because of protein above T^t (5, 26). This is a consequence of the likely tilting of the lipid chains away from the membrane normal in the vicinity of the proteins.

The experimental observations are thus in agreement with the theoretical suggestions: the boundary order parameter S_0 imposed by a protein is larger than the unperturbed value in the fluid phase and smaller in the ordered phase, and the coherence length is $\sim 15\text{\AA}$. Next we consider the influence of many such protein molecules on the lipid phase transition.

The model studied in (1) assumes a homogeneous distribution of the protein molecules in the plane of the membrane. Thus, phase separation, which is known to occur frequently below T^t , is neglected. To specify the cross-sectional area of a protein coplanar with the membrane surface, proteins are treated as cylinders. Finally, S_0 is assumed to be temperature independent. The result obtained for the spatially averaged order parameter \bar{S} is shown in Fig. 3. Three qualitative effects of protein on the ordered-fluid transition can immediately be deduced: (a) a broadening of the phase transitional region, (b) a decrease of the latent heat, and (c) an upward or downward shift of the transition temperature (including the case of constant T^t). These effects have been observed experimentally for a variety of proteins. They are consequences of our model of a "continuous boundary lipid" and may be compared with the consequences of other models commonly used to interpret experimental results.

In the model of boundary lipids that are decoupled completely from the unperturbed lipids, the latent heat (per mole of total lipid) would decrease because of protein, but the width of the transition region and the transition temperature would remain constant. If only phase separation between protein and lipid in the ordered phase is considered, the transition entropy would be increased by the mixing entropy leading to an increase of the latent heat and a decrease of the transition temperature. Since these models are too restrictive, they are not able to explain the experimental results. Note furthermore that the broadening of the phase transitional region in our model does not correspond to a decrease in cooperativity, since the number of interacting particles is not reduced by the boundary conditions.

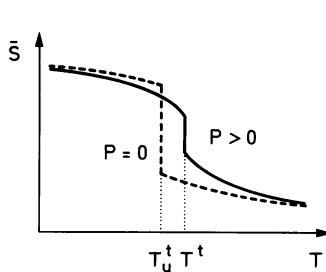


FIGURE 3

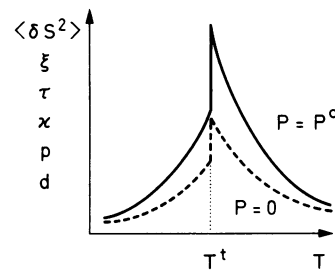


FIGURE 4

FIGURE 3 Influence of proteins (or cholesterol) on the ordered-fluid transition. With increasing protein content P the spatially averaged order parameter \bar{S} changes less at the phase transition, the phase transitional region appears broader, and the transition temperature T^t may be shifted upward or downward relative to the unperturbed transition temperature T_u^t .

FIGURE 4 Influence of proteins (or cholesterol) on the pretransitional effects. The increase of the dynamic quantities specified in the legend to Fig. 2 is enhanced by proteins and becomes maximal at the critical protein concentration P^c . The transition temperature is assumed to remain constant.

The quantitative analysis in the preceding paper (1) led to the result that the latent heat decreases linearly with increasing protein concentration, independent of the boundary condition S_0 . The transition temperature is also linearly dependent on the protein concentration, but additionally on S_0 : if S_0 lies exactly between the unperturbed order parameters of the ordered and fluid phase (at T^t), then T^t remains constant; if S_0 is larger, T^t is increased, and for smaller S_0 , T^t is decreased. At a certain protein concentration the latent heat vanishes which implies that the ordered fluid transition becomes a critical point. The critical protein concentration is determined by the coherence length ξ^t at T^t , and the molecular areas of lipid and protein in the plane of the membrane. Since the coherence length and the lipid area do not vary much for different lipids, lipid-protein systems are specified mainly by the protein area described by the protein radius R_0 . Assuming as typical values $\xi^t = 15 \text{ \AA}$ and for the lipid area 60 \AA^2 , the critical protein/lipid mole ratio is given by $P^c/L = 1/[18(1 + 2R_0/15)]$, with R_0 in \AA . For a protein radius of $R_0 = 15 \text{ \AA}$, one obtains $P^c/L = 1/54$.

Experimental results from calorimetry are available for the Ca/Mg-ATPase in two different lipid membranes (27). The phase transition temperature does not vary with protein content, whereas the latent heat decreases linearly with increasing protein content and vanishes for $P^c/L = 1/45$ in the case of dimyristoyl phosphatidylcholine (DMPC) and for $P^c/L = 1/42$ in the case of dipalmitoyl phosphatidylcholine (DPPC). These values are in good agreement with our theoretical estimate based on a protein radius of $R_0 \approx 15 \text{ \AA}$. This radius, given a thickness of 35 \AA for the hydrophobic part of the bilayer, corresponds to a molecular weight of 15,000 for the part of the protein in the hydrophobic region. The Ca/Mg-ATPase is supposed to traverse the membrane in 8 α -helices (28), which by assuming 20 amino acid residues per α -helix crossing the hydrophobic region corresponds to a molecular weight of 16,000. Thus the assumption $R_0 \approx 15 \text{ \AA}$ is realistic for the Ca/Mg-ATPase and the agreement between theory and experiment not accidental. The slight variation of the critical lipid/protein ratio between DMPC and DPPC membranes is conceivable in our model if the α -helices of the ATPase are assumed always to be incorporated in the hydrophobic region of the bilayer. Since the thickness of the latter increases upon going from DMPC to DPPC, the protein radius would have to be decreased slightly leading to a small increase of P^c/L as observed.

In the case of cholesterol, the transition temperature again remains constant and the latent heat vanishes at a molar concentration of $\sim 20\%$ cholesterol corresponding to a critical mole ratio of $Ch^c/L = 1/4$ (29). To reconcile this with the theoretical estimate we have to take into account that the rigid part of the cholesterol molecule is only about half as long as a lipid chain. This fact can be simulated by increasing our result for the critical concentration by a factor of 2. Then in the most favorable case $R_0 = 0$ we obtain $Ch^c/L = 1/9$, which is of the correct order of magnitude, but still too small by a factor of 2. This indicates that in our theory the effect of cholesterol is overestimated. Actually such a small molecule may participate to some extent in the microscopic fluctuations of the lipid chains, which would imply S_0 to be no longer constant at T^t .

Fluctuations and Response

The approach to a critical point is expressed most drastically in the dynamic behavior of the system: thermal fluctuations and response functions increase. Therefore upon addition of

protein or cholesterol the pretransitional increase of order parameter fluctuations is further enhanced. In Fig. 4 this is illustrated for the strength of the fluctuations, their coherence length and relaxation time. The same holds for the response functions specific heat, lateral compressibility, permeability, and lateral diffusion coefficient. Such effects are visible most clearly if the transition temperature remains constant. Then the fluctuations and response functions increase with protein or cholesterol concentration at constant temperature and become maximal at the critical concentration.

Quantitatively, I found (1) that the pretransitional effects increase linearly with protein concentration up to the critical point. Experimentally, when protein is added, a marked increase of the specific heat on both sides of the phase transition is found, accompanying the decrease of the sharp latent heat peak (27, 30). The same behavior is observed with cholesterol (29). Monolayer experiments indicate that cholesterol shifts the phase transition towards the critical point, which is reached at $Ch^c/L \approx 1/3$, and this shift is accompanied by an increase of the lateral compressibility on both sides of the transition (31). The corresponding decrease of the sound velocity in aqueous dispersions because of cholesterol has not been observed, instead a slight increase was found (32). This might be attributed to the use of small vesicles, where the ordered-fluid transition is altered due to steric constraints.

For the experimental study of the relaxation time of order parameter fluctuations we refer to dielectric relaxation measurements. When cholesterol was added, the dielectric relaxation time was found to increase and to become maximal at $Ch^c/L \approx 1/3$ (33, 34). This behavior indicates a critical slowing down of order parameter fluctuations. The effect was observed above T^i , but not below, which may be related to phase separation between lipid and cholesterol in the ordered phase. FA relaxation times are not much affected by cholesterol as expected for the relaxation of chain fluctuations (24, 35). Similarly, the relaxation time of C—D bond fluctuations obtained from deuterium spin-lattice relaxation is only slightly decreased by protein (5).

The permeability of a lipid membrane for small particles was found to be increased by protein (36). The same effect was observed with cholesterol (37), under the supposition that the permeation of a negatively charged dye molecule is rate limited by the permeation of small counterions. Measurements of the lipid lateral diffusion coefficient with the nuclear spin-echo technique showed an increase of the diffusion when cholesterol was added, up to a maximum at $Ch^c/L \approx 1/10$ (38). That the maximum did not occur at $Ch^c/L \approx 1/4$ may reflect the influence of boundary lipids which are expected to diffuse much slower than lipids further away from the proteins. Their increasing contribution may lead to a decrease of the average diffusion before the critical point is reached. It should be mentioned that photobleaching experiments showed a slight decrease of the lateral diffusion with increasing cholesterol concentration (39). The difference between the two experimental results has been attributed to different length scales over which diffusion is observed (38), the photobleaching technique measuring diffusion over larger distances (40) than the spin-echo technique. According to this argument the latter would detect preferentially lipid-lipid interactions and therefore be more appropriate for a study of the enhanced dynamics of the lipid phase upon addition of cholesterol.

A final remark on membrane fluidity should be added. Proteins or cholesterol increase the order of a lipid membrane in the fluid state and increase the permeability and lateral

diffusion. This dualism indicates that a membrane can not be described sufficiently by only one property such as fluidity. Static order and dynamic behavior have to be distinguished.

CONCLUSION

I have been able to show that a large number of experimental results on the ordered-fluid transition in pure lipid membranes, lipid-protein membranes, and lipid-cholesterol membranes can be interpreted consistently within the framework of a general theory for phase transitions, which has already been very successful in describing other phase transitions. The important point has been the correlation between static and dynamic properties in the vicinity of a phase transition. The static order of a lipid membrane changes at the phase transition from high to low order, whereas thermodynamic fluctuations and response functions increase on both sides of the phase transition. The smaller the change in static order at the phase transition, the more enhanced are the pretransitional dynamic effects. Critical points furnish the best example. Because proteins and cholesterol shift the ordered-fluid transition towards a critical point, they enhance the lipid dynamics. This is the reason for the increase of lateral compressibility, permeability, and lateral diffusion of a lipid membrane upon addition of proteins or cholesterol.

These theoretical predictions will, it is hoped, provoke further experimental studies, especially on the pretransitional effects at the ordered-fluid transition and their increase because of proteins. The interpretation given to existing experimental results could moreover be further extended to observations made with more complicated biological membranes (41). An interesting question in this context would be to ask whether the 30% cholesterol found in many biological membranes serves to shift the membrane towards a critical point.

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