Review Letter

INTRINSIC PROTEIN–LIPID INTERACTIONS

Physical and biochemical evidence

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1. Introduction

The present consensus view of biomembrane structure is that a lipid bilayer is the basic matrix into which and around which the various proteins are situated. Not only can the proteins be attached to the outside of the lipid bilayer (the extrinsic proteins) but in many cases the proteins (the intrinsic proteins) are embedded within and can span the bilayer.

Associated with this consensus view of biomembrane structure is the idea that in many, but not in all, cases the lipid matrix is in a fluid condition where the lipids are essentially above their T_c transition temperature and able to diffuse along the bilayer length. Not all biomembranes are highly fluid of course, and some contain crystalline lipid at the growth temperature of the cell. Many cell membrane systems (those without cholesterol) can undergo crystallisation of their biomembrane lipids when they are cooled slowly to lower temperatures [1,2].

Abbreviations: DMPC, dimirystoylphosphatidylcholine; DPPC, dipalmitoylphosphatidylcholine; DPPG, dipalmitoylphosphatidylglycerol; DSPG, distearoylphosphatidylglycerol; T_c, lipid transition temperature; PLA, myelin proteolipid apoprotein; ESR, electron spin resonance; NMR, nuclear magnetic resonance; BDH, β-hydroxybutirate dehydrogenase; PC, phosphatidylcholine; PE, phosphatidylethanolamine; DPH, 1,6-diphenyl-1,3,5-hexatriene

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The realisation that intrinsic proteins occur within the lipid bilayer structures leads directly to the question as to the extent to which the presence of such a protein perturbs its lipid environment and the significance which this may have on biomembrane structure and function.

An understanding of the molecular basis of lipid fluidity characteristics, e.g., the *gauche–trans* isomer rotations of the lipid chains, led directly to suggestions about the inhibition of these processes by cholesterol and by proteins [3]. Early calorimetric and NMR studies of biomembranes were discussed in terms of intrinsic protein–lipid perturbations rather than of the then accepted Danielli–Davson–Robertson model [4,5].

Following these early ideas other workers have developed more sophisticated concepts concerning the perturbation of the layer of lipid adjacent to intrinsic proteins.

Various terms have been used to describe this perturbed lipid, including boundary-layer lipid [6], halo lipid [7,8] and annulus lipid [9]. In all these views it is assumed that a rigid or immobilised lipid shell exists separating the hydrophobic intrinsic protein from the adjacent fluid bilayer regions. It is suggested by some workers that this shell is a single rigid lipid layer [6]; Hesketh et al. [10] go further and argue that this single rigid lipid shell is long-lived and excludes cholesterol. They argue that the rate of exchange between annular lipid and bulk lipid is slow even when the bulk lipid is fluid.

Other workers have suggested that the extent of the perturbation extends to three layers or shells of

lipid [11] and others that it extends to the sixth or seventh lipid layer [12] surrounding the particular intrinsic protein being considered.

In this Review Letter we examine the physical experimental evidence now available on intrinsic protein–lipid interactions. We also discuss critically the various attempts which have been made to relate this with the biochemical data. In particular, we examine the present evidence relating to the concepts of boundary layer lipids and annulus lipids.

2. Intrinsic proteins in biomembranes

In recent years, two main types of membrane proteins have been described, respectively, extrinsic or peripheral, and intrinsic or integral proteins. The former are readily solubilized by relatively mild methods; e.g., changes in ionic strength, while the latter require harsher procedures. Intrinsic proteins are those which are associated with the biomembranes in such a way that require the use of detergents, organic solvents or chaotropic agents to be dissociated from them; they are usually associated with lipid after solubilization, and frequently aggregate in neutral aqueous buffers.

Evidence for the presence of intrinsic proteins in biomembranes is supported by the presence of 'particles' on the fracture surfaces of native membranes and reconstituted systems. The identity and composition of these particles is still however a matter of discussion. Studies of the *Halobacterium halobium* system comparing electron diffraction and freeze-fracture pictures, show that a simple interpretation of each 'particle' as a protein is not justified [13]. These authors suggest that each 12 nm 'particle' actually consists of about 12 protein molecules, i.e., 84 transmembrane α-helices. In the case of sarcoplasmic reticulum Ca^{2+}-ATPase, it has been suggested that the hydrophobic ends of the enzyme chains join to form oligomers (3 or 4 per 90 Å particle) within the interior of the membrane [14].

Studies of the MN glycoprotein (glycophorin) of the erythrocyte membrane show that it contains an α-helical hydrophobic segment of 23 amino acid residues [15,16] which spans the lipid bilayer. One end of this segment carries all of the carbohydrate groups whilst the other, normally exposed to the cytoplasmic side of the erythrocyte membrane, carries charged amino acids. The outside diameter of the helical segment is in the region of 10–15 Å. A similar structure has been found with bacteriorhodopsin [17–19] in which the protein occurs as groups of α-helices that can span the membrane. Cytochrome b_5 has a structure with a hydrophobic, lipid-binding anchor, and a globular hydrophilic domain containing the active centre [20,21]. Band-3 protein of erythrocytes is believed to be a complex of glycoproteins involved in anion transport spanning the membrane [22–24].

Other examples of proteins which span the lipid bilayer of membranes are the cytochrome oxidase complex from mitochondrial inner membranes, which has been studied by electron microscopy and by labelling with subunit-specific antibodies [25], and the larger subunit of the $(Na^+\text{-}K^+)$ATPase from rabbit kidney outer medulla, which binds oubain on the outer surface and has the phosphorylation site on the inside surface of the membrane [26,27].

3. Physical studies of intrinsic molecules in lipid bilayers

In order to provide insight into the processes which occur when an intrinsic molecule is present in a lipid bilayer we shall first briefly examine what is known about the effects due to small molecules, such as cholesterol and simple polypeptides, before we examine the physical data obtained with intrinsic proteins.

3.1. *Cholesterol*

In lipid bilayer systems the effect of cholesterol upon the surrounding lipid has been studied both above and below the T_c transition temperature. Above the temperature various techniques including NMR and ESR spectroscopy as well as fluorescence spectroscopy confirm that the presence of cholesterol perturbs and modulates the lipid fluidity [28–30].

Below the lipid T_c transition temperature calorimetric studies show that the main lipid endotherm is removed with increasing amounts of cholesterol. The first studies [31] suggested that the enthalpy was totally removed at 50 mol%, whilst later studies

[32] suggested that this occurred at 33 mol%. The latter conclusions led to the concept that cholesterol existed as a 2:1 lipid–cholesterol complex [33]. The postulated structure of this complex is shown in fig.1a. It can be seen that in this model it is envisaged that each cholesterol molecule is surrounded by a lipid shell. This is reminiscent of the models proposed for proteins in model and natural biomembranes [2].

Very recent studies [34] using sensitive scanning calorimeters confirm the early conclusion that the enthalpy is in fact removed at 50 mol% of cholesterol to lipid as originally envisaged [31]. It is suggested [34] that there are two peaks superimposed on the calorimeter trace, one corresponding to the transition of patches of pure lipid and another to a broad transition associated with the cholesterol-rich phase. The results of these recent studies are inconsistent with the envisaged 2:1 lipid–cholesterol complex.

It is generally agreed that in the case of cholesterol in lipid bilayers, above the T_c transition temperature a modulation of lipid fluidity occurs, causing the lipid to be less fluid, whilst below the T_c transition temperature the lipid is prevented from crystallising. The detailed nature of lipid–cholesterol systems is still open to discussion. It has recently been suggested that a random arrangement of lipid and cholesterol might be sufficient to rationalise the existing data [35]. A diagram illustrating such an arrangement is shown in fig.1b.

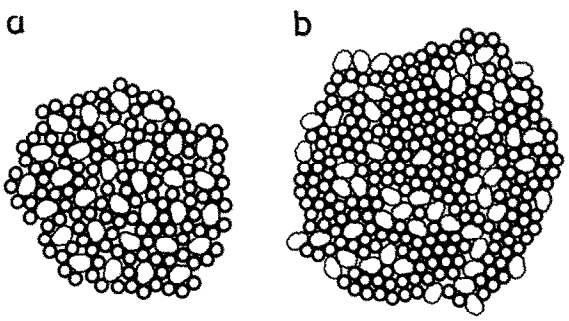


Fig.1. (a) An arrangement of lipid and cholesterol molecules corresponding to a 2:1 phospholipid–cholesterol complex as suggested by Engelman and Rothman [33]. (b) An experimentally simulated random array of lipid and cholesterol molecules corresponding to a 2:1 phospholipid : cholesterol ratio [35].

3.2. *Polypeptides*

Physical studies of the perturbation due to a hydrophobic (intrinsic) polypeptide, gramicidin A, upon the surrounding lipid bilayer have been made using a variety of physical techniques [36,37].

3.2.1. Above the lipid T_c transition temperature

Raman spectroscopic studies of gramicidin A in pure lipid bilayers show a reduction in the content of the *gauche* isomers of the lipid hydrocarbon chains as deduced from an examination of the spectral regions 100–1200 cm^{-1} and also the region near 2800 cm^{-1}. The effect due to gramicidin is greater at the same molar ratio as is observed with the cholesterol molecules.

ESR studies show that, using either fatty acid or phospholipid spin labels, at very high concentrations of the polypeptide (1 polypeptide per 5 lipid molecules), a new broad hyperfine signal is observed with cytochrome oxidase systems at high protein to lipid content and attributed to boundary layer lipid [6] whereas with the polypeptide it is attributed to 'trapped lipid' [36] (fig.2).

3.2.2. Below the lipid T_c transition temperature

Calorimetric studies of gramicidin in pure lipid water systems show that the presence of the polypeptide causes the main lipid transition to increase in width, and a loss of enthalpy to occur. It was suggested that aggregation of the polypeptides might occur.

3.3. *Proteins*

Studies have been made of intrinsic proteins in reconstituted and natural biomembranes.

3.3.1. Escherichia coli membranes

Trauble and Overath studied lipid phase transitions in *Escherichia coli* membranes using fluorescent probes [7]. They suggested (on the basis of a comparison of the amount of lipids which takes part in the phase transition with the total extracted lipids of the cell membrane) that some 20% of the lipids is removed from the phase transition process. They suggest that one 'integral membrane protein' is surrounded by about 600 lipid molecules with some 130 of these forming a halo around the protein.

Recent results by Gent and Ho [38] using ^{19}F NMR

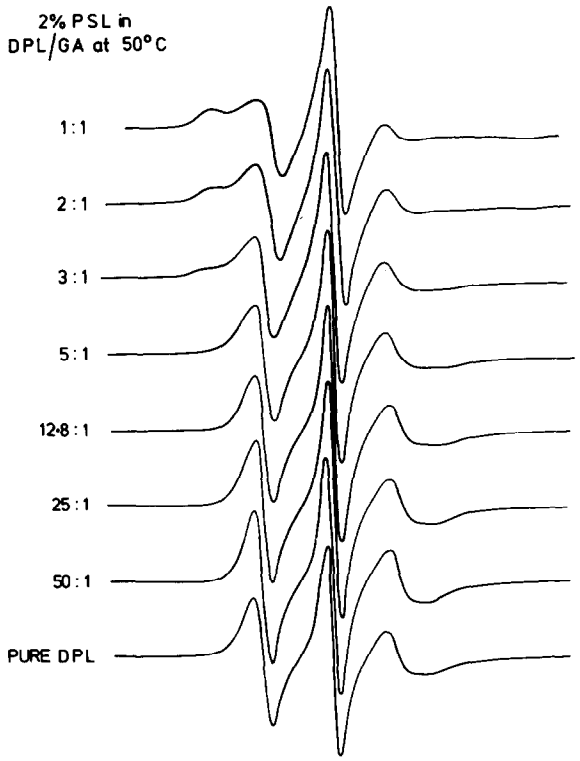


Fig.2. The ESR spectra at 50°C of 2% (w/w) phospholipid spin label in DPPC–gramicidin A dispersions with the indicated molar ratios [37].

on *E. coli* membranes suggest that, below the T_c transition temperature, two types of lipids occur: rigid crystalline and protein perturbed. The exchange between them is slow. Above the T_c transition temperature only one type of lipid is detectable by ^{19}F NMR because the lipid in contact with the protein exchanges rapidly with the bulk lipid.

3.3.2. Liver microsomal membranes

Stier and Sackmann [8] studied the effect of the cytochrome *P*-450 reductase system on two different types of spin label one being water soluble and the other lipid soluble (stearic acid nitroxide). A marked difference was observed as a function of temperature. The activation energy of the fatty acid reduction decreased abruptly at about 32°C whilst no break was observed with the water-soluble label. They interpreted their data in terms of a rigid lipid halo which is in a quasi-crystalline structure below 32°C whilst the bulk lipid of the membrane is in a fluid state.

3.3.3. Cytochrome oxidase systems

Several authors have studied spin-labelled lipids with mixtures of cytochrome oxidase and phospholipids, either natural [6] or synthetic [39]. Analysis of the ESR spectra from a series of mixtures of varying lipid : protein ratio are interpreted in terms of two distinct lipid environments for the spin probe. In one environment the probe is mobile and the spectral component is similar to that found for the probe in pure lipid bilayers. The second spectral component corresponds to a highly immobilized probe and this has been interpreted as a fraction of the lipid bilayer tightly bound to the cytochrome oxidase in a monomolecular layer. A critical assumption in the extrapolation of these data to the model of boundary lipid is that the spin probe behaviour is similar to that of the phospholipids in the bilayer. It is possible that, although a fraction of the spin probe is tightly bound to the protein, the membrane phospholipids may not partition in the same way. However, the same results were obtained using two very different spin probes, a fatty acid and a steroid probe [40]. Another source of uncertainty in the interpretation of these results is that cytochrome oxidase contains 6 subunits whose functions and relationship to the membrane are not yet fully understood [41].

Dahlquist et al. [42] studied the effects of cytochrome *c* oxidase upon the specifically deuterated lipid 1-(16^1,16^1,16^1-trideuteropalmitoyl)-2-palmitoleylphosphatidylcholine and noted that the protein converted a sizeable fraction of the lipid molecules into a more ordered component; the boundary layer.

Recent studies [43,44] do not support these conclusions and no evidence is found for the two component, bilayer plus 'boundary lipid' spectra. Instead the presence of the cytochrome oxidase causes a decrease in the quadrupole splitting (or order parameter) of the methyl group of the lipid chains. These authors argue that the cytochrome oxidase has the effect of preventing lecithin hydrocarbon chains from crystallising below the lipid T_c transition temperature whilst above T_c it causes a disordering of the lipid chains.

ESR spin label, and ^2H NMR results, for three

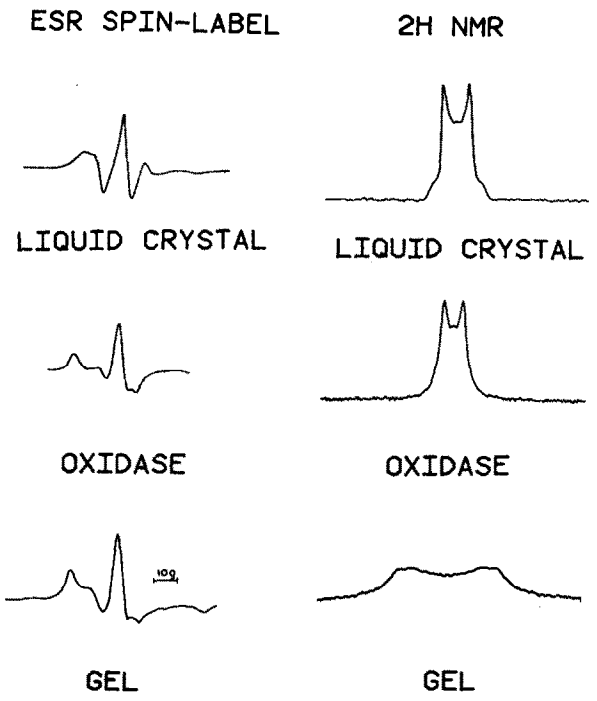


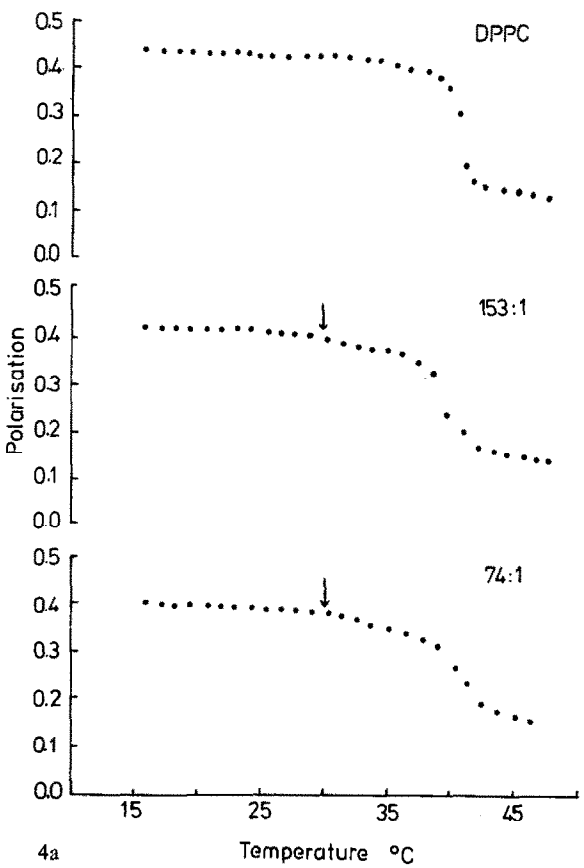
Fig.3. Comparison between ESR spin label and ^{2}H NMR view (using a terminal methyl-labelled lecithin) of hydrocarbon chain order in gel, liquid crystal and cytochrome oxidase–lipid (boundary lipid) states. Reproduced with permission from [44].

lipid states, are presented in fig.3: A pure liquid crystalline lecithin bilayer, 'boundary lipid' and lipid in the crystalline gel state. As viewed by ESR the rigid gel state of lecithin below T_c is similar in motional properties to the rigid or ordered 'boundary lipid' of cytochrome oxidase. However, as viewed by ^{2}H NMR of a terminal methyl-labelled lecithin, 'boundary lipid' is even more disordered than the lipid above its T_c transition temperature [44].

3.3.4. Sarcoplasmic reticulum ATPase

Hesketh et al. [10] have studied reconstituted complexes of sarcoplasmic reticulum ATPase with pure lipids (e.g., DPPC) using fatty acid spin-labelled molecules. They observed two component ESR spectra and attributed the presence of an 'immobile component' to the presence of a rigid lipid annulus of 30 lipid molecules which interact directly with the ATPase. These authors also state that the spin label studies show no DPPC phase transition near 41°C for lipid : protein ratios of less than 30 lipids per ATPase (their data show a transition at 40.5°C for a 29:1 lipid–protein complex) (see fig.3, Hesketh et al. [10]). The immobile ESR component is shown to be present for various complexes of different lipid : protein content (17:1–67:1) but at 30°C (i.e. below the lipid phase T_c transition temperature of 40.5°C). Another feature of the data is that for the 29:1 lipid–protein complex the immobile component reduced considerably in intensity between 30°C and 40°C and has only weak intensity at 50°C.

Recent studies have also been made on this system using calorimetric, fluorescence and ESR spin-label studies [45]. These studies show that above the lipid T_c transition temperature, as indicated by the fluorescent probe studies, the 'microviscosity' of the system increases as the concentration of the protein in the lipid increases (fig.4). The ^{2}H NMR studies



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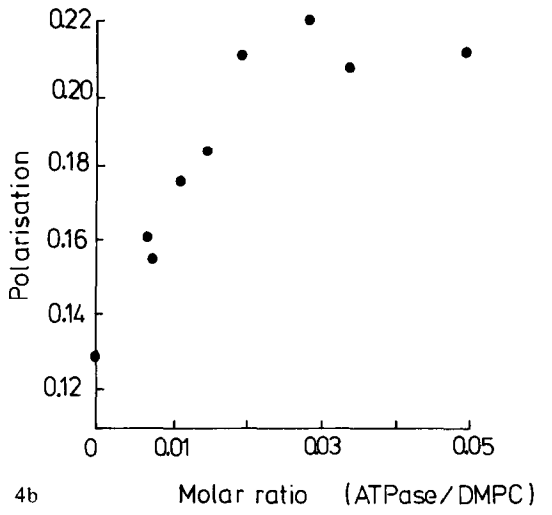

Fig.4. (a) Polarisation P of a fluorescent probe (DPH) in DPPC–water and DPPC–(Ca^{2+}-Mg^{2+})ATPase–water systems, containing defined amounts of proteins as a function of temperature. DPPC:ATPase molar ratios are indicated in each case. (b) Changes of polarisation P at a constant temperature 36°C, at various DMPC:(Ca^{2+}-Mg^{2+})ATPase molar ratios [45].

show the existence of only one component and not two components in the spectra [46].

3.3.5. Myelin proteolipid apoprotein

Recent studies have been made of the protein–lipid interactions which occur with the apoprotein of myelin in reconstituted systems with DMPC [12,47]. These authors used X ray, calorimetry and Raman spectroscopy. The X-ray data showed that incorporation of this apoprotein (PLA) into the lipid bilayer does not change the overall bilayer structure.

The Raman data showed that the presence of the apoprotein perturbs the thermal behaviour of the chains of the lipids. Above the lipid main T_c transition temperature the lipid chains are inhibited from completely attaining their normal *gauche* conformation. Below the lipid T_c transition temperature the number of *trans* chains is reduced.

The calorimetric data show that as the protein concentration is increased the pretransition peak disappears and the main peak broadens and decreases in height. A shoulder appears on the main peak at higher temperatures and at 20% PLA (w/w) only a broad peak remains (fig.5).

These authors conclude from the calorimetric data that three to four concentric layers of phospholipid around each protein molecule are perturbed and constitute the boundary layer. They suggest that the PLA exhibits a similar effect to the action of cholesterol but at lower cholesterol proportions.

3.3.6. Rhodopsin and retinal rod outer segment membranes

A number of workers have studied rhodopsin and boundary layer lipid effects associated with this

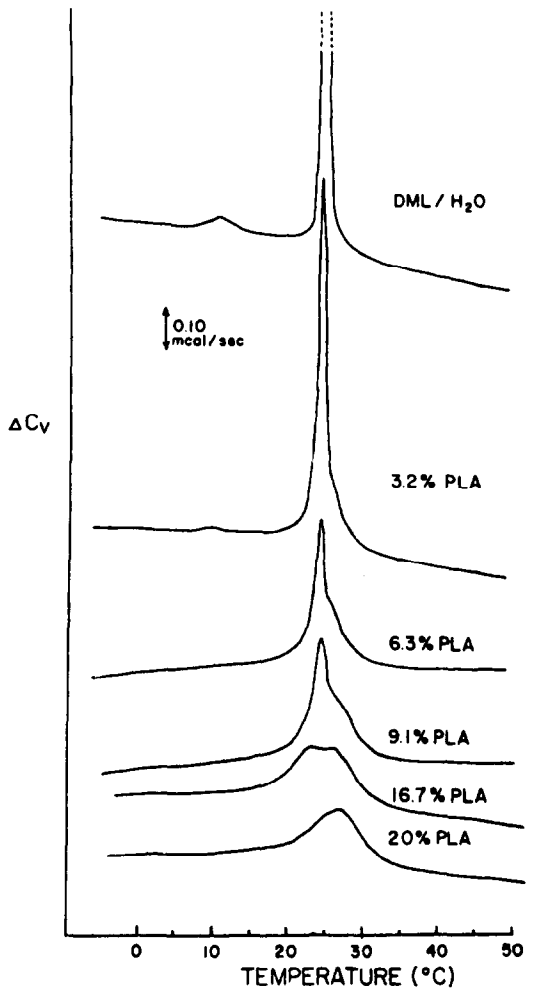

Fig.5. DSC thermograms of DMPC and DMPC/PLA recombinants. All traces shown are heating runs obtained at 1.25°C/min. Each sample contained 7–9 mg DML and varying amounts of protein. After Curatolo et al. [47].

protein. Rhodopsin was tested with spin-labelled fatty acids. Pontus and Delmelle [48] found evidence of a rigid boundary layer around this hydrophobic protein. However, Hong and Hubbell [49] had reached a different conclusion from spin-label experiments with rhodopsin reincorporated into phospholipids; they suggested that the average viscosity of the membranes was dependent on the lipid : protein ratio. This is in agreement with recent results put forward by Cherry et al. [50] from very different experiments involving bacteriorhodopsin. Using proton NMR and rod outer segment membranes, Brown et al. [51] concluded that, although the rotational motion of hydrocarbon chains was affected by the proximity of the proteins, all phospholipids can diffuse rapidly in the plane of the membrane suggesting a homogeneous lipid phase.

Devaux et al. [52,53] have recently carried out a large number of experiments using spin-label methods where they studied the immediate environment of the lipid near to rhodopsin in the disc membranes and without changing the lipid : protein ratio. They conclude that, with a hydrophobic spin-label covalently bound to rhodopsin via a hydrophobic SH group, but able to locate in its near environment, it gives rise to an ESR spectrum not very different from the spectrum of a spin-labelled fatty acid diffusing freely in the lipid bilayer, i.e., a high degree of disorder or fluidity is observed. On the other hand, when the membranes are partially delipidated using phospholipase A_2, an immobile component is always observed. A difference of 4 orders of magnitude occurs between the correlation times of the probe situated in the boundary layer of intact disc membranes and membranes with low lipid content [52].

4. Theoretical studies

There have been a number of theoretical studies to estimate the extent to which an intrinsic protein may affect the surrounding lipid in a lipid bilayer structure. Marčelja [11] has published a microscopic mean field model of order in lipid bilayers based on chain conformation. Schroeder [54] described a method of incorporating lipid–protein interactions into a pre-existing mean field treatment of lipid bilayers. The protein acted formally as an external field on the lipids. In both the above studies the attractive lipid-mediated interaction between two identical proteins was demonstrated.

Jähnig [55] also has developed a microscopic mean field bilayer model based on chain conformation. Owicki et al. [56] studied the order perturbation as a function of temperature and lateral pressure using Landau-de Genne's theory and a variational procedure. They conclude that for a given lateral pressure there is a temperature dependence and that the greater amount of boundary layer is present at the main lipid T_c-transition temperature.

Pink and Chapman [57] have used a lattice model and examined lipid systems where the proteins interact only via Van der Waal's interactions and also systems where the proteins have bound or attached lipids on their circumference. These calculations have been used to examine the melting temperatures of eutectic protein–lipid patches.

5. Biochemical studies

The natural amphiphiles associated with membrane proteins, that usually prevent them from aggregation and denaturation, are the phospholipids. An exposure to a hydrophilic medium leads to protein aggregation.

For some proteins, it has been claimed that a minimum amount of lipids per protein is necessary for maintaining full enzyme activity. This is the case of Ca^{2+}-ATPase reconstituted with pure lipids, as well as associated to endogenous sarcoplasmic reticulum lipids [10,58]. About 30 lipid molecules per ATPase has been found to be the minimum ratio. This is interpreted as a lipid annulus [10]. In the case of cytochrome *c* oxidase, 0.2 mg lipid/mg protein appears to be essential for keeping the enzyme activity after removal of the other lipids by acetone extraction [59]. These and other results have been interpreted in terms of the necessity for a shell of lipids surrounding the enzyme in order to maintain its activity. This interpretation has been applied to Ca^{2+}-ATPase [9], cytochrome *c* oxidase [6] and BDH [60], reconstituted with pure, synthetic lecithins. In general, lipids associated to shorter-chain fatty acids give lower enzyme activities.

The enzyme activity does not completely dis-

appear below the T_c lipid transition temperature. This has been shown for a variety of reconstituted enzymes: Ca^{2+}-ATPase with DPPC [10], (Na^+-K^+) ATPase with DPPG and DSPG [61], and BDH with DPPC [60]. One interpretation assumes the presence of an annulus of lipid around the proteins, whose physical properties are different from those of the bulk lipid. An alternative suggestion for this is given in section 6 in terms of eutectic mixtures.

The role of lipids in the regulation of membrane protein function could include as well the control of some physical parameters affected by phase transitions, e.g., the hydration of the interphase, as has been suggested for C_{55}-isoprenoid alcohol phosphokinase [62].

5.1. *Lipid specificity*

No lipid specificity with respect to polar groups has been shown for several enzymes, like (Na^+-K^+) ATPase [63], C_{55}-isoprenoid alcohol phosphokinase [64] or cytochrome *c* oxidase [65]. A more complex picture is found in sarcoplasmic reticulum ATPase. It has been claimed [66] that PC lipids support ATPase better than PE lipids whereas Ca^{2+} translocation activity required for maximal rates both PC and PE lipids.

There are a few examples in which polar groups of phospholipids are connected to enzyme activity because they act like 'allosteric efectors' producing a large increase in the enzyme activity by enhancing ligand binding (substrate or coenzyme). Such is the case of BDH and phosphatidylcholine: the cofactor NADH is not bound except in the presence of the lipid [67]. Something similar is the case of pyruvate oxidase from *E. coli* [68]. It has been also suggested that cardiolipin interaction with some components of mitochondrial ATPase may regulate its activity [69]. Some other purified membrane enzymes have been examined for their preferences for polar groups like phosphoenolpyruvate phosphotransferase from bacterial membranes which would require phosphatidylglycerol for optimal activity [70]; glucosyl [71] and galactosyltransferases [72] from *Salmonella typhimurium* require PE lipids. But Dean and Tanford [73] have shown that phospholipids can be fully substituted with exogenous, synthetic amphiphiles, e.g., detergents, without loss of enzyme activity with the sarcoplasmic reticulum ATPase.

6. Discussion

It seems to us that it is necessary to try to be clear first about the physical perturbation effects of intrinsic proteins within a lipid bilayer structure. Some authors have tried to group together this physical perturbation effect with enzymatic effects associated with 'minimum' lipid, 'residual' lipid and 'specific' lipid characteristics.

There are, in our opinion, three important points to take into account:

(i) The time scale appropriate to the particular physical technique used to study the protein–lipid perturbation. This can be appreciated when we realise that, measured on one time scale, say 10^{-8} s using ESR spin-labelled molecules, a molecule may appear to be rigid, whilst on another time scale, say 10^{-5} s using ^2H NMR methods, the same molecule can appear to be mobile. When attempts are made to relate some measured perturbation effects with enzymatic effects then yet another time scale must be considered, i.e., the time interval over which the enzymatic conformational effect occurs.

(ii) The concentration of the protein and its arrangement within the lipid bilayer which is being examined. When we look at the plane of the lipid bilayer (see fig.6) it is immediately

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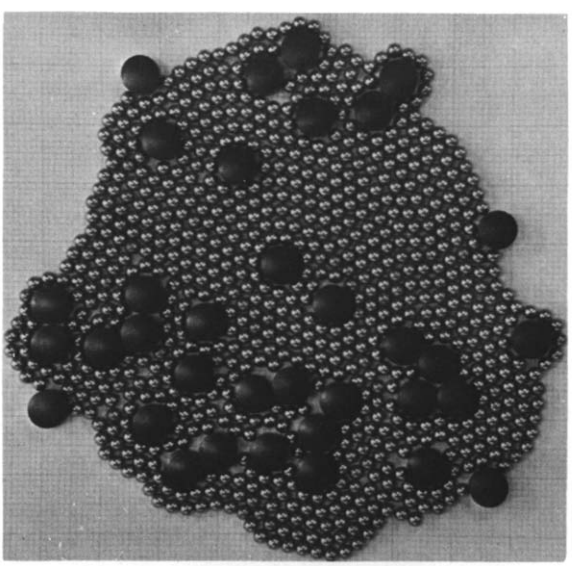

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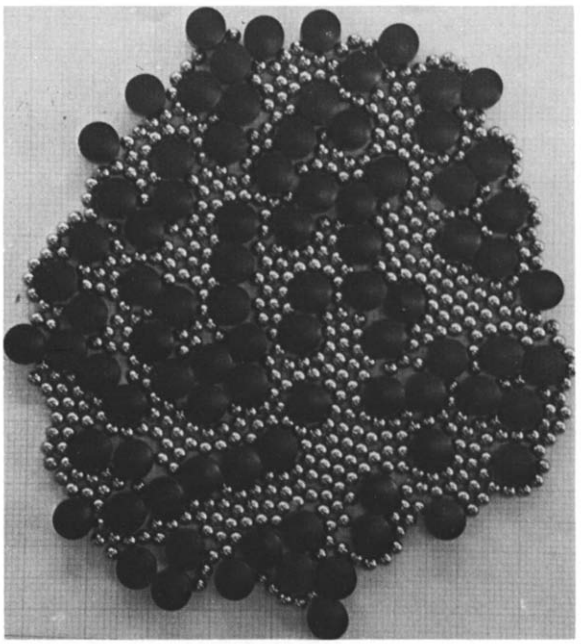

Fig.6. Simulated lipid–polypeptide arrays with lipid : polypeptide ratios of (a) 10:1 and (b) 3:1 [37].

apparent that as the concentration of the protein in the lipid bilayer increases the number of multiple contacts of each lipid with proteins also become important. At low protein concentrations single contacts of lipid with the protein are the dominant situation.

(iii) Whether in the system considered the lipid is above or below its T_c transition temperature. The lipids below the T_c transition temperature, when they crystallise, squeeze the proteins out of the crystalline lipid lattice. The shape and size of the protein causes packing faults (see fig.7) in the lattice and patches are formed of high protein : lipid content, i.e., eutectic mixtures occur. These regions have been demonstrated by freeze-fracture electron microscopy and are distinct from the remaining crystalline lipid regions. A simulation of this is shown in fig.8. The lipid within the patches may be prevented from crystallising. These lipids can be rigid because of the high protein content present. They will become mobile at temperatures below the lipid T_c transi-

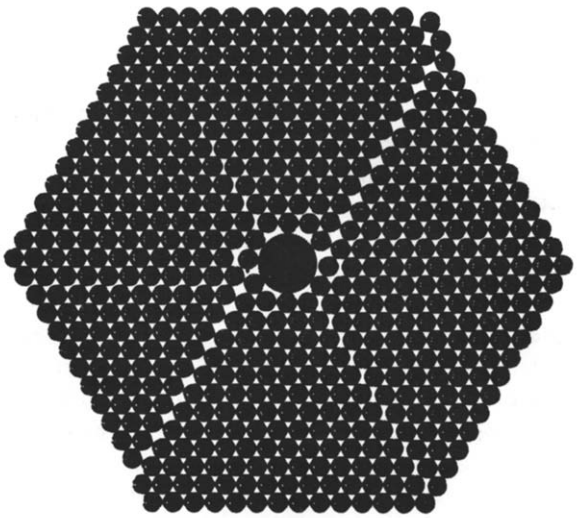

Fig.7. Typical pattern of packing faults resulting from introduction of a cylinder of non-critical size into a ball-bearing raft. Ball bearings represent hydrocarbon chains of 48 Å diameter. Cylinder is 120 Å in diameter. Packing faults lie along hexagonal axes of closely-packed array of ball bearings [75].

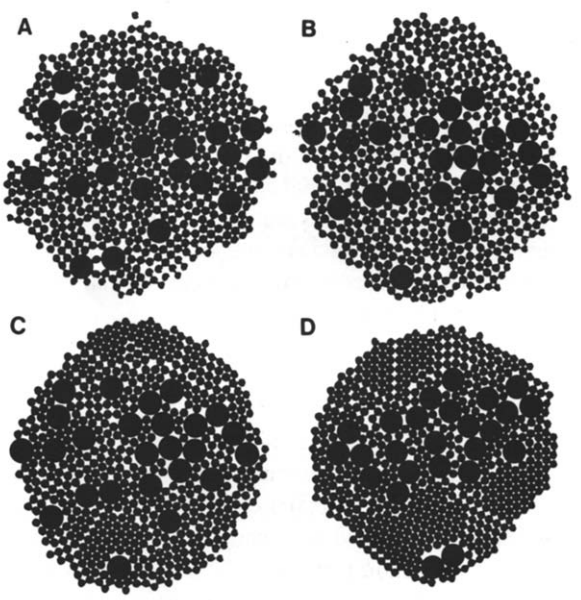

Fig.8. Schematic diagram showing how lipid crystallisation can lead to protein aggregation: (A) random distribution of lipid and protein; (B) more extensive areas of lipid nucleate-producing domains of crystalline lipid intersected by packing faults seen in (C) the crystallisation of certain areas of lipid forces protein molecules to aggregate; (D) equilibrium low temperature distribution of lipid and protein [75].

tion temperature the remaining crystalline lipid will melt and disaggregation of the patches will occur.

With thse considerations in mind, we now attempt to rationalise the various data.

The variously published Laser-Raman spectroscopy studies show that intrinsic proteins and polypeptides cause a reduction (on a time scale of $\sim 10^{-14}$ s) of the lipid hydrocarbon *gauche* isomers at concentrations of about 1 intrinsic polypeptide per 200 lipid molecules. [12,36]. (More studies are required to be certain that this is a general phenomenon.)

Fluorescent probe studies indicate that the motion of the probe molecule becomes inhibited as the protein (or intrinsic polypeptide) concentration increases [45]. This indicates that the fluidity of the lipid decreases or that the 'microviscosity' increases (see fig.4). Consistent with this conclusion are the studies of Devaux et al, on protein rotation of rhodopsin using saturation transfer ESR methods [53], as well as studies by Cherry et al. [50] using laser flash photolysis and reconstituted protein from *Halobacterium halobium* (see section 3.3).

ESR experiments with cytochrome oxidase and sarcoplasmic reticulum ATPase (time scale $\sim 10^{-8}$ s) have shown the existence of 'immobile components' in the corresponding spectra (section 3.3). It has been proposed that the ESR immobile component is an indication of a special lipid shell (called the boundary layer lipid or the annulus lipid) separate and distinct from the bulk lipid. The annulus lipid is said to control enzyme activities of intrinsic proteins [10].

Other workers have pointed out a number of observations which cast doubt upon this interpretation of the observed ESR 'immobile' component and of the concept of special annulus lipid.

(1) It has been shown that the observation of such an ESR 'immobile' component is not restricted to proteins which may possess captive or bound lipids. It is indeed observed in gramicidin A–lipid–water systems [36]. The gramicidin A molecule is a relatively simple polypeptide.

(2) The immobile ESR component is observed at high protein (or polypeptide) content in the lipid bilayer [36]. At such high protein or polypeptide contents, the microviscosity will be high. Mobility of the probe molecule will be expected to be considerably inhibited and multiple contacts of lipid with protein (trapped lipid) will occur [37].

(3) In ESR experiments of rhodopsin [52] using spin-labelled molecules which are attached to the protein but penetrate and sense the boundary layer, little effect on the mobility of the probe is found. This shows that a high degree of fluidity exists in the boundary-layer phospholipids. However when the membranes are delipidated an 'immobile' ESR component is observed. They further conclude that lipid depleted membranes cannot be used to characterise the viscosity of the boundary layer of native membranes.

(4) NMR studies using either ^{1}H, ^{19}F or ^{2}H nuclei (time scale 10^{-3}–10^{-5} s) on various biomembranes or reconstituted systems do not show the occurrence of two types of lipid. This is the case with ^{1}H NMR studies of rhodopsin in disc membranes [51], with ^{19}F NMR studies of *E. coli* membranes [38] and ^{2}H NMR studies of the cytochrome oxidase and sarcoplasmic reticulum systems [44,46].

Thus a continuity between the bulk lipid phase and the boundary layer lipids and ready diffusion between these lipids takes place, as indicated by the NMR experiments.

In relation to this apparent discrepancy between the ESR and NMR data, we believe [57] that the polar group packing of the lipids and proteins is an important factor to be considered in these systems. When we consider a phospholipid lying adjacent to an isolated protein, the effect of one on the other may not be too large because there are many other polar groups associated with other lipids next to the phospholipid being considered. The effect of the protein on adjacent lipid is thus a two-bodied effect. In this case, the fluidity decreases with increasing protein concentration in the fluid state.

As protein concentration increases, proteins can trap the lipid between them (see fig.6). In this case, the polar group of the phospholipid is not next to other lipid polar groups, but next to protein polar sections. This then becomes a three-bodies effect, proportional to the protein concentration.

This means that at any temperature a lipid chain might be more fluid than is the pure-lipid–water system itself.

Recent ^{2}H NMR studies of polypeptide, protein and cholesterol in lipid bilayer systems [74] are

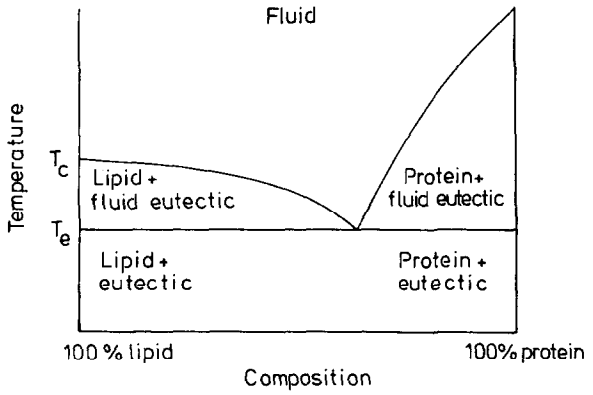

Fig.9. Schematic phase diagram of lipid–protein mixtures. T_e is the eutectic temperature and T_c corresponds to the main transition temperature of the pure lipid.

consistent with these concepts.

We have pointed out earlier that when the temperature of a reconstituted system is lowered below the lipid T_c transition temperature, the lipid chains crystallise, proteins are squeezed out and eutectic mixtures of protein and lipid are formed (see fig.9). This has significance for a number of situations:

(i) The aggregation process produces two regions, one consisting of crystalline lipid, relatively free of protein, the other consisting of patches or protein–lipid aggregates. As yet more protein is included to the lipid system, the aggregated patch increases in size at the expense of the remaining crystalline region. This is why lipid–water systems show a broadening of the main melting transition and a reduction of the enthalpy as protein is incorporated. The reduced enthalpy value may correspond to an average perturbed lipid. The reduction of the enthalpy observed with some biomembranes [7] e.g., *E. coli* has been interpreted as a measure of the boundary layer or halo lipid. It is assumed that this is the same as the amount of perturbed lipid which occurs above the lipid T_c transition temperature, and the formation of eutectic mixtures of high protein to lipid content has not been considered.

(ii) Probe molecules such as ESR or fluorescent probes in the lipid bilayer system may tend to be concentrated in these protein–lipid patches. Upon raising the temperature, a marked increase of mobility below the lipid T_c transition temperature will occur, i.e., at the T_e eutectic 'melting' temperature. This is shown in fig.4 where the polarisation P of DPH shows a marked effect at 30°C for the DPL–ATPase-reconstituted system.

(iii) The enzymatic activity of a number of reconstituted enzymes increases considerably below the transition temperature T_c of the pure phospholipid. This is observed with the $(Na^+\text{-}K^+)$ATPase in synthetic phosphatidylglycerols [61] and with the sarcoplasmic reticulum ATPase incorporated into DPPC systems [10] (in the latter case at 30°C whereas the main T_c transition temperature of the lipid is 41°C). When there is sufficient crystalline lipid (free from protein) remaining, a disaggregation process will occur due to the melting of the separate crystalline lipid at the T_c transition temperature and a further effect may occur on the enzyme mobility.

(iv) Some authors have commented upon the fact that a minimum amount of lipid is required of optimum enzymatic activity and attempted to relate this to the presence of a special lipid annulus. However, a minimum amount of lipid may be required merely to retain the appropriate fluidity for the protein to operate, and to prevent irreversible protein aggregation.

7. Conclusions

The fact that an intrinsic protein may perturb its surrounding lipid is clear. The type of perturbation depends, however, on the concentration of protein within the lipid bilayer. At low protein concentrations, an ordering of lipid chains can occur whilst at high protein concentrations a disordering effect occurs. At these high concentrations the perturbation of the lipid polar group packing has to be considered.

The time scale appropriate to the particular physical technique used to study the protein–lipid perturbation must be related to the time scale of the phenomenon being examined.

Concepts such as boundary-layer lipids or annulus lipids, implying long-lived shells of lipid controlling

enzyme activity, and only slowly exchanging with fluid lipid, are not supported by recent NMR experiments. The ESR data showing an 'immobile component' should be reconsidered in terms of other effects, e.g., due to high microviscosity of trapped lipid, the specific attachment of the ESR probe to the protein, or lipid inside eutectic mixtures.

The formation of aggregates or eutectic mixtures of high protein to lipid content is an important consideration when reconstituted systems and membranes are studied at temperatures below the T_c transition temperature of the pure lipid.

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