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HYDROCARBON PHASE TRANSITIONS AND LIPID-PROTEIN INTERACTIONS IN THE ERYTHROCYTE MEMBRANE

A ^{31}P NMR AND FLUORESCENCE STUDY

P.R. CULLIS * and Ch. GRATHWOHL

Biochemistry Department, South Parks Road, Oxford OX1 3QU (U.K.)

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Summary

1. ^{31}P NMR and other techniques have been employed to study various derivatives of the human erythrocyte ghost membrane, derived liposomes and liposomes composed of mixtures of synthetic phosphatidylcholines. It is shown that the previously reported (Cullis, P.R. (1976) FEBS Lett. 68, 173—176) phospholipid phase transition observed in ether extracted ghosts is also observable employing fluorescence depolarization techniques. This phase transition can be (reversibly) removed by re-addition of cholesterol.

2. A new semi-biological model membrane system is described which may be obtained by proteolytic digestion of peripheral membrane protein and a subsequent ether extraction. Freeze-etch studies and other considerations indicate that these "pronase digested ether extracted ghosts" consist of segments of integral membrane protein surrounded by one or two boundary layers of lipid.

3. Within the terms of the "fluid mosaic" model of biological membranes lipids may exist in at least four different environments. These include unperturbed bilayer regions, bilayer regions shielded by peripheral membrane protein, lipid experiencing strong polar interactions with membrane protein (bound lipid), and lipid associated with integral membrane protein (boundary lipid). The results obtained here are consistent with the following general conclusions.

(a). The ether extraction procedure removes most, if not all, of the unperturbed bilayer lipid.

(b). The amount of membrane phospholipid "bound" to membrane protein so as to cause immobilization in the phosphate group region (on the time scale of 10^{-5} s) is at most 3% of the membrane phospholipid.

(c). Phospholipids in all three of the major classes of lipid (unperturbed

* Present address: Biochemistry Department, Transitorium 3, "De Uithof", Padualaan 8, Utrecht, The Netherlands.

bilayer, shielded bilayer and boundary lipid) exhibit ^{31}P NMR spectra consistent with bilayer structure.

(d). The motion (on the time scale of 10^{-5} s) in the phosphate group region of membrane phospholipids is not sensitive to the presence of peripheral membrane protein.

(e). The phase transition behaviour and ^{31}P NMR lineshapes observed suggest that in the ether extracted ghost system phospholipids in bilayer regions (possibly consisting largely of sphingomyelin) enter the gel state below 20°C. Below 20°C "boundary" phospholipids also experience restricted motion. Above 20°C the results would be consistent with a model whereby boundary phospholipids experience fast exchange with lipids in liquid crystalline bilayer regions.

Introduction

In a recent investigation it was shown that ether-extracted human erythrocyte ghosts exhibit a phospholipid phase transition in the region of 20°C [1]. Thus, as the ether extraction procedure removes all the cholesterol from the membrane [2] it was suggested that the phase transition observed in normal erythrocyte ghosts by other techniques [3,4] corresponded to a transition of a fraction of lipids in cholesterol-poor regions of the membrane. However, it was also noted that the transition temperature (T_c) of the phospholipids of the ether extracted ghosts was more than 15°C higher than the transition temperature of the extracted lipids in model membrane liposome systems, and there were also marked differences between ^{31}P NMR lineshapes and linewidths obtained from the model and protein containing systems. It was suggested that such effects must be due to the influence of membrane protein, although no precise interpretation was attempted.

The ether-extracted membrane is therefore a most interesting membrane system, as the effects of lipid-protein interactions would appear to have marked effects on the motional properties of the constituent phospholipids which are directly observable. In the present study we have therefore extended the previous investigation to more accurately characterize the nature of the lipid protein interactions involved. A particular attempt has been made to approach the problem from the viewpoint of the "fluid mosaic" [5] model of biological membranes. In this regard, the ether extraction procedure is of interest, as incubation of the biological membrane in a solvent with such a low dielectric constant would be expected to dramatically strengthen polar interactions and weaken apolar interactions, as compared to the situation when the membrane is hydrated. Thus, it would be expected that ether extraction would remove lipids in bilayer regions which were not shielded by peripheral membrane protein, which is indeed consistent with the results obtained here. Such speculations also suggest a further logical experiment however, in that removal of extrinsic protein prior to ether extraction should result in a marked increase in the amount of lipid extracted. Such an experiment has been performed in this work, resulting in a system which appears to be comprised solely of integral membrane protein and one or two layers of boundary lipid. Comparative studies of this system and the ether-extracted ghost may then be used to derive

information on the properties of lipids in bilayers and boundary environments.

In this work we have employed primarily ^{31}P NMR techniques, which have been previously shown to be sensitive to the motional and structural properties of phospholipids in membranes [6—10]. In particular instances fluorescence depolarization studies have been performed. The results obtained are discussed in terms of the fluid mosaic model of biological membranes.

Materials and Methods

Erythrocyte ghosts and ether-extracted erythrocyte ghosts were prepared from outdated human bank blood in the manner detailed previously [1]. Pronase (ex *Streptomyces griseus*, Koch-Light Labs. Ltd., Colnbrook, Bucks, U.K.) digestion treatments were performed according to the procedure of Rottem et al. [11]. Total lipids were extracted from the various erythrocyte ghost preparations using the method of Bligh and Dyer [12] with 100 mM NaCl and 50 mM EDTA present in the aqueous phase. Protein was determined by the method of Lowry [13], and phospholipid phosphorus by the Fiske-SubbaRow procedure [14]. The egg-yolk phosphatidylcholine, dioleoyl phosphatidylcholine and dimyristoyl phosphatidylcholine were a kind gift of Dr. B. de Kruijff, whereas dipalmitoyl and distearoyl phosphatidylcholine were obtained from Koch-Light and Calbiochem, San Diego, Calif., respectively. The ether employed for the ether extraction was dried over KOH pellets and sodium. All other reagents were of analar grade and were used without further purification.

Liposomes were prepared from a chloroform solution of the required lipids where the chloroform was evaporated under N_2 so that a thin film of the lipid was deposited on the bottom of a glass vial. After at least 1 h storage under high vacuum, 1 ml of H_2O containing 25 mM Tris/acetic acid (pH 7.2) was added, and liposomes were formed by agitating the vial on a vortex mixer at a temperature at least 5°C higher than the highest transition temperature of any of the component lipids.

The 129 MHz Fourier Transform ^{31}P NMR spectrometer employed in this investigation was built in this laboratory [15]. This spectrometer was interfaced with a Nicolet B-NC 12 computer, and was equipped with temperature control and quadrature detection facilities. Accumulated free induction decays were obtained from up to 5000 transients, employing a 60° pulse and 0.5 s interpulse time.

Fluorescence depolarization measurements were performed on an instrument built in this laboratory [16] employing the hydrophobic fluorescent dye 1,6-diphenyl-1,3,5 hexatriene (obtained from Koch-light). The fluorescence was excited at 360 nm and detected at 450 nm after being passed through a filter with a cut-off at 420 nm. The fluorescence depolarization was monitored continuously as the sample was heated through 0—40°C. Temperature control was achieved via a regulated bath. The diphenyl hexatriene was added to the aqueous membrane suspension in a tetrahydrofuran medium such that an approximate molar ratio of 1 : 500 of dye to phospholipid was achieved. After addition of the dye the solution was gently agitated for 0.5 h at 20°C to permit equilibrium partitioning of the dye into the membrane.

A ^{31}P NMR technique was devised to ascertain whether a significant fraction

of erythrocyte membrane phospholipid was bound to membrane protein so as to cause immobilization in the phosphate region of the polar headgroup. The spectral intensity (i.e., the integral of the signals of the phospholipid spectra) is directly proportional to the amount of contributing phospholipid, in the absence of saturation effects. If, however, the phosphate group is immobilized due to lipid-protein interactions, very broad ^{31}P NMR lines ($\Delta\nu_{1/2} \geqslant 100$ ppm) would be expected, which would not be detected. Thus the spectral intensity of the signals arising from erythrocyte ghost membranes was calibrated with respect to the spectral intensity of a standard egg-yolk phosphatidylcholine liposome preparation (which has a similar lineshape and width, thus minimizing possible instrumental artifacts). Subsequently, equal volumes of perchloric acid were added to both the ghost and standard samples, which served to totally disrupt and disperse the membrane. Then, the intensity of the (denaturated) erythrocyte sample was again calibrated with respect to the standard. In this case it may be presumed that all the phospholipid phosphate of the biological sample is contributing to the observed ^{31}P NMR signal. A comparison of the intensity of the denaturated membrane to that of the intact membrane then allows the amount of non-observable, or bound phospholipid to be determined. Such a procedure was found to give reproducible results within 2%.

Freeze fracture studies were performed as described previously [17].

Results

The protein:phospholipid ratios, phospholipid composition and the amount of "non-observable" phospholipid which characterize erythrocyte ghosts after various treatments are given in Table I. It may be noted that the phospholipid composition does not vary dramatically between normal ghosts, ether-extracted ghosts and pronase digested ether extracted ghosts with the exception of the high concentration of sphingomyelin and low concentration of phosphatidyl-

TABLE I

PROTEIN : LIPID RATIO AND LIPID COMPOSITION OF ERYTHROCYTE GHOSTS AFTER VARIOUS TREATMENTS

Material	Protein : phospholipid ratio (by wt.)	Phospholipid composition (expressed as percentage of total phospholipids)				Amount of phospholipid not observed by ^{31}P NMR
		Phosphatidylcholine	Sphingomyelin	Phosphatidylethanolamine	Phosphatidylserine	
Normal ghost	1.5	31	23	31	14.5	⩽3%
Pronase-digested ghosts	0.4					
Ether-extracted ghosts	2.5	30	47	13.7	9	⩽3%
Ether-extracted, pronase-digested ghosts	0.64					
Pronase-digested, ether-extracted ghosts	1.6	32	34	21	11.6	

ethanolamine found in the ether-extracted system. The latter results are in some disagreement with the results of Roelofsen et al. [2] and may arise because of ATP depletion of the erythrocytes from which the ghosts were derived. It has been shown recently that such ATP depletion causes a marked increase in the amount of phosphatidylethanolamine that may be extracted under ether (for chicken erythrocyte ghost), relative to the amount of sphingomyelin extracted [18]. The protein:phospholipid ratios show that the pronase digestion procedure results in the removal of 72% of the membrane protein from both the normal ghost and ether-extracted membrane system. Alternatively, the ether extraction removes 40% of the membrane phospholipids from normal erythrocyte ghosts, whereas ether extraction of the pronase-digested ghost results in the removal of 75% of the membrane phospholipids. Finally, the amount of phospholipid "bound" such that ^{31}P NMR signals could not be observed was found to be less than 3% of the total phospholipid for both the normal and ether-extracted ghost systems. In order to further characterize the pronase digested, ether-extracted ghost system freeze-etch studies were also made. As shown in Fig. 1 this system appears to consist solely of very densely packed intramembrane particles. It should be noted that such particles are present on both the inner and outer fracture faces. The density of these particles may be estimated to be approximately $10^4/\mu m^2$.

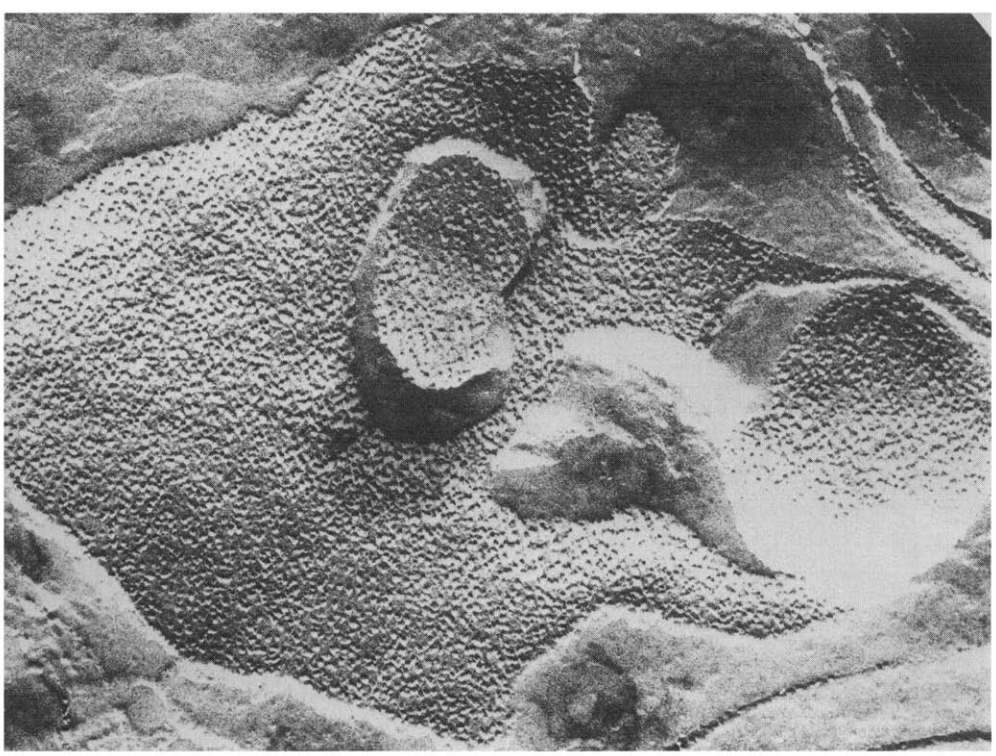

Fig. 1. Freeze-fracture electron micrograph of pronase-digested, ether-extracted ghosts rehydrated in 25 mM Tris/HAc (pH 7.2). The sample was quenched from 0°C. Magnification 93000 ×.

As previously indicated, we suggest that the appearance of the gel-liquid crystalline phospholipid phase transition at 20° C in the ether-extracted ghost membrane arises primarily because of the removal of cholesterol, as it is known that the presence of cholesterol inhibits phase transition phenomena in both model [19] and biological membranes [20,21]. In order to demonstrate this more conclusively, cholesterol was re-introduced into a (lyophilized) ether-extracted ghost membrane preparation by incubating it in ether saturated with respect to cholesterol. As shown in Fig. 2, subsequent ^{31}P NMR studies indicate that the phase transition at 20° C is not observed. Moreover, a subsequent ether-extraction of the reintroduced cholesterol resulted in the reappearance of the phospholipid phase transition at 20° C.

The ^{31}P NMR phase transition behaviour of the phospholipids remaining in the pronase-digested, ether-extracted ghosts is illustrated in Fig. 3. It may be noted that a phase transition at 20° C is observed, similar to that observed for the ether extracted system. It is also shown that liposomes composed of lipids extracted from the pronase digested ether extracted membrane do not undergo such transition behaviour.

In order to characterize the phase transition of the lipids of the ether-extracted ghost membrane more closely, the fluorescence depolarization of the hydrophobic fluorescent dye diphenyl hexatriene (which is sensitive to the fluidity in the hydrocarbon chain region [22]) was studied. The results obtained for normal ghosts, ether-extracted ghosts and liposomes composed of lipids extracted from these systems are indicated in Fig. 4. It may be noted that only the ether-extracted ghost preparation exhibits the characteristic sigmoidal shape associated with hydrocarbon phase transition phenomena [16,22]. The transition temperature as estimated from the first differential is 23.5 ± 2° C,

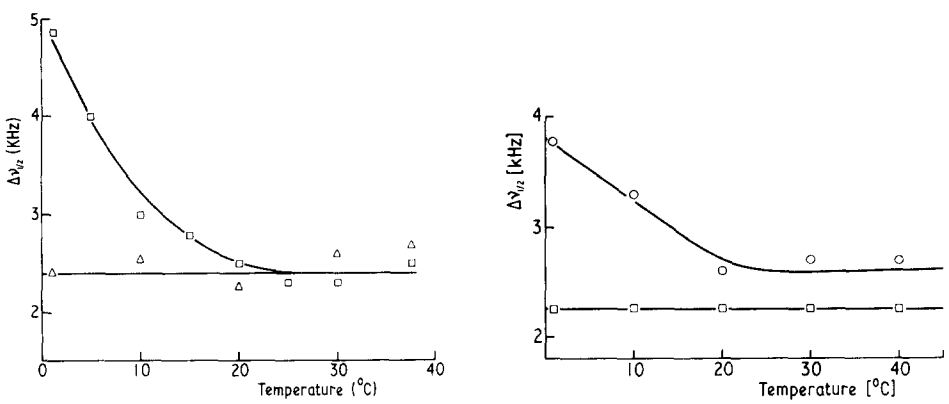

Fig. 2. Temperature dependence of the width ($\Delta\nu_{1/2}$) of the 129 MHZ ^{31}P NMR spectra obtained from ether-extracted erythrocyte ghosts (□) and ether-extracted erythrocyte ghosts after incubation in ether saturated with cholesterol (△). Both systems were rehydrated with 25 mM Tris/HAc (pH 7.2).

Fig. 3. Temperature dependence of the width ($\Delta\nu_{1/2}$) of the 129 MHz ^{31}P NMR spectra obtained from pronase-digested, ether-extracted ghosts (○) and liposomes of the derived lipids (□). Both systems were rehydrated in 25 mM Tris/HAc (pH 7.2).

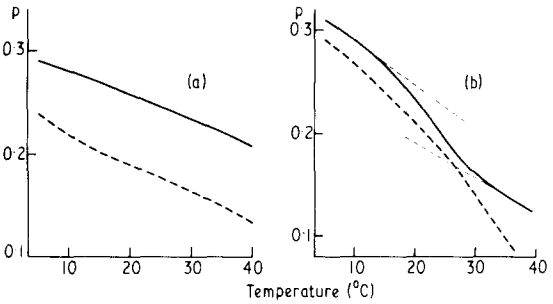

Fig. 4. Temperature dependence of the fluorescence depolarization (P) of DPH introduced into erythrocyte ghosts and derived liposomes. (a) Solid curve, normal erythrocyte ghosts; dashed curve, liposomes composed of extracted lipids. (b) Solid curve, ether-extracted erythrocyte ghosts; dashed curve, liposomes composed of the extracted lipids. All suspensions contained 25 mM Tris/HAc (pH 7.2).

and the width of the transition may be estimated to be approximately 14°C. These results therefore indicate that the ^{31}P NMR technique which indicates a phase transition at 20°C, is sensitive to the low temperature end of the phase transition.

As indicated in the next section, lipids in biological membranes may be considered to belong to at least three different categories: lipids in unperturbed lipid bilayer regions, lipids in bilayer regions protected by peripheral membrane protein, and boundary lipids closely associated with integral membrane protein. Effects such as hydrocarbon phase transitions, which reflect the cooperative effects of lipid-lipid interactions, would not be expected to occur for boundary lipids [23]. Thus, only a certain fraction of the lipids in the derivative erythrocyte ghost membranes may undergo phase transitions, and it would be expected that these lipids are sequestered away from the region of integral membrane proteins. The boundary lipid, on the other hand, may be considered to be somewhat immobilized as a result of lipid-protein interactions. Further, the fluorescence depolarization studies indicate that in the bilayer regions conditions of lateral phase separation must occur in the region 20—30°C. Thus, at least three motionally distinct types of phospholipid may be present, immobilized boundary lipid, immobilized gel state lipid and liquid crystalline lipid.

In order to ascertain the effects that might be expected when membrane systems contain fluid liquid crystalline regions and less fluid gel or boundary layer lipid, the ^{31}P NMR characteristics of selected liposome systems consisting of mixtures of synthetic phosphatidylcholines in which lateral phase separation may occur were studied. The temperature dependence of the linewidths ($\Delta\nu_{1/2}$) of the ^{31}P NMR spectra arising from equimolar mixtures of 14 : 0/14 : 0 and 18 : 0/18 : 0 phosphatidylcholine, 18 : 1_c/18 : 1_c and 16 : 0/16 : 0 phosphatidylcholine, and 18 : 1_c/18 : 1_c and 18 : 0/18 : 0 phosphatidylcholine are illustrated in Fig. 5. It is known from calorimetric studies [24] that equimolar aqueous mixtures of 14 : 0/14 : 0 and 18 : 0/18 : 0 phosphatidylcholine may exhibit lateral phase separation. The phase transition temperature T_c of the 14 : 0/14 : 0 phosphatidylcholine component is increased to approx. 30°C, whereas the T_c of the 18 : 0/18 : 0 phosphatidylcholine is decreased to approx.

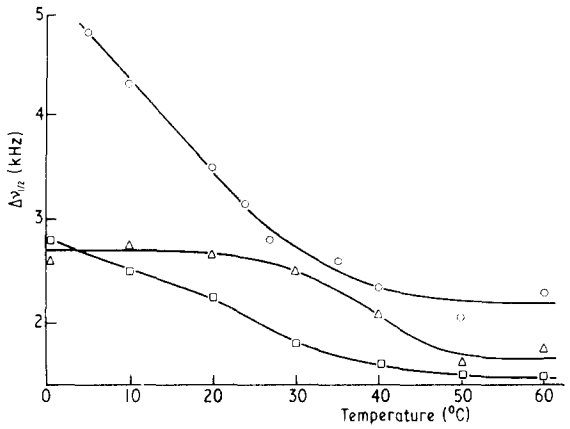

Fig. 5. Temperature dependence of the width ($\Delta\nu_{1/2}$) of the 129 MHz ^{31}P NMR spectra obtained from liposomes composed of equimolar amounts of 14 : 0/14 : 0 and 18 : 0/18 : 0 phosphatidylcholine (○), equimolar amounts of 16 : 0/16 : 0 and 18 : 1_c/18 : 1_c phosphatidylcholine (□) and equimolar amounts of 18 : 1_c/18 : 1_c and 18 : 0/18 : 0 phosphatidylcholine (△). All suspensions contained 25 mM Tris/HAc (pH 7.2).

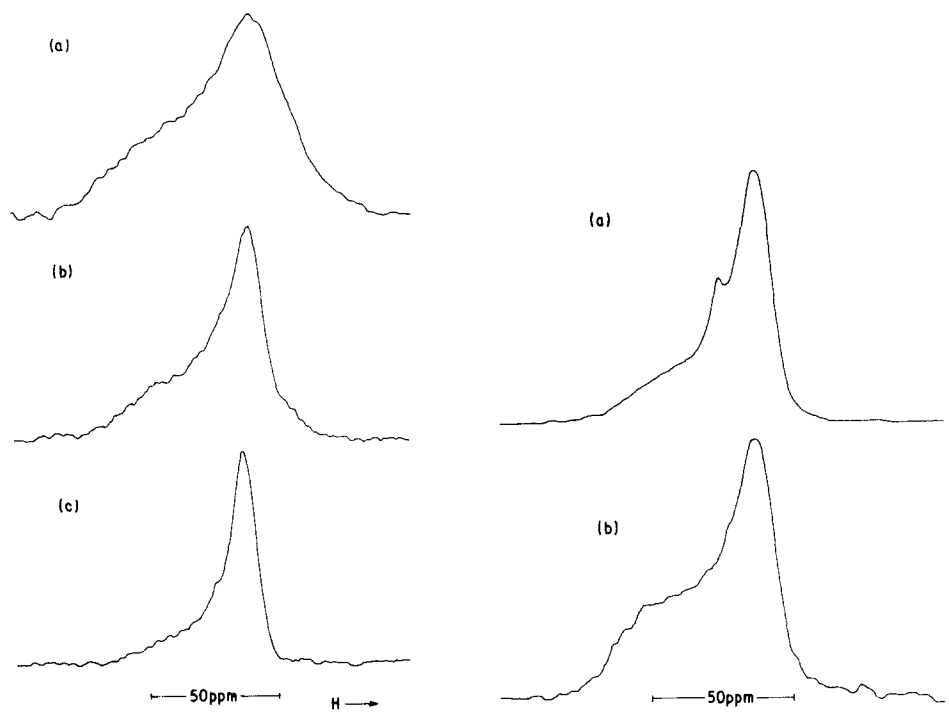

Fig. 6. 129 MHz ^{31}P NMR spectra obtained from: (a), an equimolar mixture of 14 : 0/14 : 0 and 18 : 0/18 : 0 phosphatidylcholine at 5°C (where both lipid species are in the gel state); (b), an equimolar mixture of 18 : 1_c/18 : 1_c and 16 : 0/16 : 0 phosphatidylcholine at 10°C (where lateral phase separation between gel state 16 : 0/16 : 0 phosphatidylcholine and liquid crystalline 18 : 1_c/18 : 1_c phosphatidylcholine occurs); (c), an equimolar mixture of 18 : 1_c/18 : 1_c and 16 : 0/16 : 0 phosphatidylcholine at 50°C (where both lipid species are in the liquid crystalline state). All lipid dispersions contained 25 mM Tris/HAc (pH 7.2).

Fig. 7. 129 MHz ^{31}P NMR spectra obtained at 30°C from (a) fully hydrated 14 : 0/14 : 0 phosphatidylcholine and (b) partially hydrated (20% by wt.) 14 : 0/14 : 0 phosphatidylcholine.

45°C [24]. Alternatively, for the mixtures containing 18 : 1_c/18 : 1_c phosphatidylcholine, the transition temperature of the 18 : 1_c/18 : 1_c phosphatidylcholine remains unchanged at −20°C, whereas the T_c of the 16 : 0/16 : 0 phosphatidylcholine is reduced to 30°C and the T_c of the 18 : 0/18 : 0 phosphatidylcholine is reduced to 45°C [24]. In all cases the phase transition of the species with the highest melting temperature are markedly broader. As noted in Fig. 5 for the 18 : 0/18 : 0—14 : 0/14 : 0 phosphatidylcholine mixture the ^{31}P NMR spectra obtained broaden dramatically when both phospholipid species enter the gel state. Alternatively, for the mixtures containing 18 : 1_c/18 : 1_c phosphatidylcholine (where lateral phase separation occurs) the linewidths remain constant until the temperature approaches the transition temperature of the species with the highest value of T_c, whereupon a further narrowing occurs.

Another parameter which is of interest concerns the ^{31}P NMR lineshape obtained. As seen in Fig. 6, the low field shoulder is much more distinct for liposomes in which lateral phase separation occurs (Fig. 6b) than for liposomes composed of purely liquid crystalline phosphatidylcholine (Fig. 6c). Another situation which can also produce similar marked changes in lineshape is illustrated in Fig. 7 for two preparations of liquid crystalline 14 : 0/14 : 0 phosphatidylcholine with differing water content. It may be noted that the partially hydrated (20% by wt.) system exhibits a much more well defined low field shoulder than does the fully hydrated system.

Such differences in lineshape are also apparent between the ^{31}P NMR spectra obtained from erythrocyte ghost, ether-extracted ghost and pronase-digested, ether-extracted ghost phospholipids in the intact systems and in the derived liposome systems. Thus, as may be noted on comparison of the spectra of Figs. 8 and 9 the low field shoulder is far more prominent in the protein-containing systems (Fig. 8) than in the derived liposomes (Fig. 9).

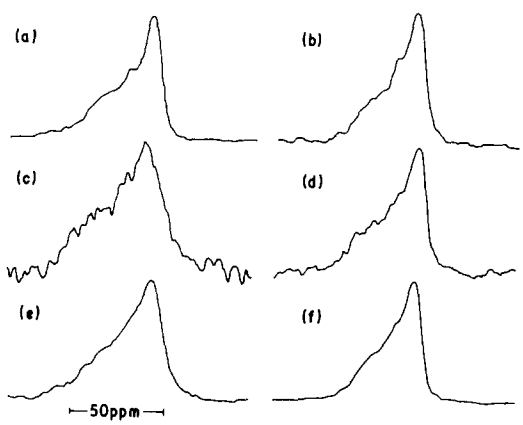

Fig. 8. 129 MHz ^{31}P NMR spectra obtained from erythrocyte ghosts and various derivatives at 2°C and 37°C: (a) and (b), normal erythrocyte ghosts, T = 2°C and 37°C respectively. (c) and (d), ether-extracted erythrocyte ghosts, T = 2°C and 37°C respectively; (e) and (f), pronase-digested, ether-extracted ghosts, T = 2°C and 37°C respectively. All membrane suspensions contained 25 mM Tris/HAc (pH 7.2).

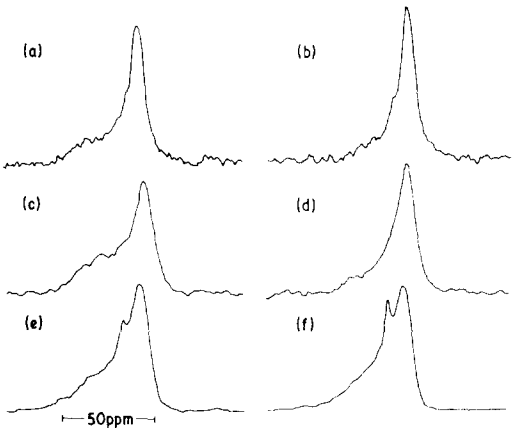

Fig. 9. 129 MHz ^{31}P NMR spectra of liposomes composed of lipids extracted from erythrocyte ghosts and derivatives: (a) and (b), liposomes derived from normal erythrocyte ghosts, $T = 2°C$ and $37°C$ respectively; (c) and (d), liposomes derived from ether-extracted erythrocyte ghosts, $T = 2°C$ and $37°C$ respectively; (e) and (f), liposomes derived from pronase-digested, ether-extracted ghost, $T = 2°C$ and $37°C$ respectively. All membrane suspensions contained 25 mM Tris/HAc (pH 7.2).

Discussion

Within the terms of the fluid mosaic model of biological membranes [5] lipids may be classified as belonging to one of at least three different categories: unperturbed bilayer regions, lipids in bilayer regions covered by peripheral protein ("shielded" bilayer lipid) and lipids in the vicinity of integral membrane proteins ("boundary" lipid). The studies presented here give direct information of (a) the occurence, (b) the structure and (c) the motional properties of phospholipids in each of these classes.

Before examining these details, however, the relation between the fluid mosaic model and the ether extraction procedure is of interest. The ether extraction would be expected to remove those lipids which do not have the possibility of experiencing polar interactions with associated protein and thus would be expected to remove most, if not all, of the lipid in the unperturbed bilayer regions. This proposal is consistent with previous observations [18] and also with the results obtained here. As noted in the freeze-etch results of Fig. 1, the pronase-digested, ether-extracted ghost system only contains very densely packed intramembrane particles. The ether-extracted ghost system, on the other hand, contains regions of bilayer structure (Verkleij, A., unpublished results). Thus the ether-extracted ghost membrane lipid appears to exist in shielded lipid bilayer and boundary lipid regions, whereas the pronase-digested, ether-extracted ghost membrane lipid may be considered to be primarily boundary lipid.

The proposal that the pronase-digested, ether-extracted system contains primarily boundary lipid and integral membrane protein is also supported by the protein:phospholipid ratio of 1.6 (Table I), which is rather large for a membrane which may be considered to contain only integral protein. If it is assumed that these intramembrane particles (of approx. 8.5 nm diameter [25, 26]) constitute the only protein, and that these particles traverse the mem-

brane, the protein:lipid ratio of the system where only one boundary layer of lipids is present may be calculated. For reasonable values of area/lipid molecule (50 $Å^2$), membrane thickness (40 Å) and protein density (0.9 g/ml) the protein-boundary lipid ratio in such a system is approximately 2 (w/w). Thus, the pronase-digested, ether-extracted system, with a protein:lipid ratio of 1.6, would appear to consist of segments of integral protein surrounded by one, or two, layers of boundary lipid. Such a system obviously has great potential in the elucidation of lipid-integral protein interactions.

The amount of phospholipid in particular regions may be estimated, assuming that the ether extraction removes all the lipid in unshielded bilayers. The results of Table I indicate that unperturbed bilayers contain 40% of the membrne phospholipid, whereas 35% of the phospholipid is in shielded bilayer regions and the remaining 25% constitutes the boundary lipid. The relative amounts of shielded and unshielded bilayer lipid would appear to be sensitive to ATP depletion of the erythrocytes prior to preparation of the ghosts [18], however.

With regard to the structure and motion of the phospholipids in the erythrocyte membrane and in the various derivatives, the following general conclusions may be drawn. Firstly, in all the systems investigated the ^{31}P NMR spectra obtained are consistent with the restricted anisotropic motion expected for phospholipids in a bilayer configuration (refs. 8 and 27 and Cullis, P.R., McLaughlin, A.C. and Hemminga, M.A., unpublished). No evidence is found for lipids in non-bilayer configurations (such as hexagonal or cubic phases) which give markedly different ^{31}P NMR spectra [8]. Further, the maximum amount of phospholipids which may experience strong polar interactions with membrane protein resulting in immobilization in the phosphate group region on the time scale of 10^{-5} s is at most 3%. Secondly, the motion in the phosphate group region of the phospholipids is not sensitive to the presence of peripheral membrane protein. This was demonstrated previously [1] for the erythrocyte ghost and ether-extracted ghost membrane, and is also compatible with results obtained for rat erythrocyte ghosts labelled with the phosphonium derivatives of choline, where it was shown that the motion in the $P^+(CH_3)_3$ region of phosphonium phosphatidylcholine is also insensitive to the removal of periperal protein [28]. Thus it would appear likely that the motional properties of phospholipids in unshielded and shielded bilayer regions of the erythrocyte membrane are very similar. A third conclusion is that the presence of cholesterol in the native erythrocyte ghost system is directly responsible for inhibiting phase transition phenomena. It thus appears that cholesterol plays a similar role in the erythrocyte membrane as in model membrane systems, where it has been shown that cholesterol "condenses" phospholipids above their transition temperature, and "fluidizes" them at temperatures below T_c [19].

The phase transition behaviour of the derivative erythrocyte membrane systems may be employed to give further information on the interactions of phospholipids with integral membrane protein. The observation of the ether-extracted ghost membrane phase transition by fluorescence depolarization techniques suggests that the ^{31}P NMR technique detects, at 20°C, the low temperature end of a hydrocarbon phase transition. Hydrocarbon phase transition effects reflect the cooperative effects of lipid-lipid interactions, which are not

expected to occur for boundary lipid [23]. Thus, the observed phase transition must be occuring in lipids in shielded bilayer regions in the ether extracted ghost membrane. As the removal of the peripheral membrane protein does not affect the transition behaviour [1] it would appear that this transition is a natural property of the bilayer phospholipids themselves. Differential scanning calorimetry results [29] indicate that only sphingomyelin exhibits such a high phase transition temperature where the low temperature end of the sphingomyelin phase transition occurs at approx. 20°C and the transition is at least 15°C wide. Thus, it may be suggested that in the ether-extracted ghost membrane sphingomyelin is segregated into bilayer regions, and that other species of phospholipid experience preferential interactions with integral protein below 20°C. In order to resolve this possibility, we are currently investigating the transition properties of ether-extracted ghost systems derived from different animal species which contain markedly different amounts of sphingomyelin (ref. 30 and Grathwohl, Ch. and Cullis, P.R., in preparation).

Information on the motional effects of these lipid-protein interactions is also available. It should be noted that ^{31}P NMR is not sensitive to the actual phospholipid hydrocarbon phase transition, but rather is sensitive to the effects of the hydrocarbon phase transition on the dipolar interactions experienced by the phosphate phosphorus (Cullis, P.R., McLaughlin, A.C. and Hemminga, M.A., unpublsihed). There is strong evidence which suggests that effects are due to restriction of rapid axial rotation ($\tau_r \geqslant 10^{-5}$ s) of the phospholipid below the phase transition (Cullis, McLaughlin and Hemminga, unpublished). Thus, ^{31}P NMR would not be expected to be able to discriminate between phospholipids in the gel state, and phospholipids whose motion was restricted by other factors (such as interactions with proteins). It should be noted that the ^{31}P NMR results do not indicate precisely how much of the membrane phospholipid enters a state motionally equivalent to the gel state below 20°C. However, the results obtained for the model systems consisting of mixtures of synthetic phosphatidylcholines indicate that much more than 50% of the membrane phospholipids must enter a gel-like state below 20°C in order to obtain the pronounced line broadening effects noted at low temperatures. As sphingomyelin constitutes less than 50% of the phospholipid in the ether-extracted ghost membrane, and only 34% of the lipid in the pronase-digested, ether-extracted ghost membrane, it must be concluded that boundary phospholipid is also relatively immobilized below 20°C.

Above 20°C two different interpretations of the observed effects are possible. It may be suggested that boundary lipid remains immobilized and does not exchange with surrounding (liquid crystalline) lipid. However, such a model is not consistent with the very similar ^{31}P NMR lineshapes and phase transition behaviour observed for the ether-extracted ghost and pronase-digested, ether-extracted ghost systems. On the basis of this model spectra characteristic of gel phospholipids would be expected over the entire temperature range investigated for the pronase-digested, ether-extracted ghost membrane, due to the far higher proportion of boundary lipid. We therefore propose that above 20°C the boundary lipids are able to exchange rapidly (on the time scale of 10^{-5} s) with nearby liquid crystalline lipids. As the temperature is decreased through 20°C we suggest that sphingomyelin is preferentially excluded from the region of

the integral protein, either as a result of preferential lipid-protein interactions with more fluid phospholipid species, or as a result of the preference of sphingomyelin for lipid-lipid interactions with other gel state sphingomyelins. Thus, the available pool of liquid crystalline phospholipids for the boundary lipids to exchange with would be depleted leading to ^{31}P NMR spectra characteristic of gel state phospholipids from both the gel state sphingomyelin and the boundary phospholipid.

The ^{31}P NMR lineshapes obtained for the protein-containing membranes above 20°C are of interest, particularly when compared to the spectra obtained from the derived liposomes. As noted in Figs. 8 and 9 the liposomes composed of the extracted lipids exhibit ^{31}P NMR spectra which are much narrower and which have a more poorly defined low field shoulder than do their protein-containing counterparts. These results indicate that the presence of (integral) membrane protein introduces an ordering effect among the phospholipids, reducing the motion available in the phosphate group region. Such effects likely reflect a reduction in the area per lipid molecule in the presence of protein which may arise from hydrophobic interactions with integral membrane protein or from polar lipid-protein interactions which reduce the hydration of associated lipids.

In summary, studies have been performed on erythrocyte ghosts which have been subjected to a logical series of progressive perturbations, and the results obtained can be interpreted in terms of the fluid mosaic model of biological membranes. Within the framework of this model, the most important implications of the present work are that boundary lipids may exchange rapidly (on a time scale of 10^{-5} s) with surrounding (lipid crystalline) lipids, that gel state phospholipids are preferentially excluded from the region of integral membrane protein, that the motional properties of all classifications of phospholipid are consistent with bilayer structure, that the presence of integral membrane protein produces an ordering effect on associated lipids and that peripheral membrane protein does not markedly affect the motional properties of underlying phospholipid. It should be noted however that these general conclusions relate to macroscopic effects observed in poorly defined systems. It may be expected that quite different behaviour occurs in particular instances, an understanding of which will require careful study of much more carefully defined lipid-protein model membranes.

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