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THE INFLUENCE OF PROTEIN-LIPID INTERACTIONS ON THE ORDER-DISORDER CONFORMATIONAL TRANSITIONS OF THE HYDROCARBON CHAIN

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Summary

The phases of simple systems involving one type of protein (lysozyme or cytochrome *c*) and one type of lipid (phosphatidic acid) have been characterized by X-ray crystallography, chemical analysis and spin-labeling technique as a function of temperature. They are of the lamellar type with alternative protein monolayers and lipid bilayers. According to the pH, two types of lamellar phases are obtained, one where the lipid-protein interactions are mainly hydrophobic, the other where they are electrostatic. In both cases, a phase transition occurs as temperature is lowered, between a high temperature phase, where all the lipids are in the liquid-like state, and another phase where some lipid chains are rigid. In the case of the phases with electrostatic interaction, it is shown that the onset of the order-disorder transition is shifted towards low temperature as compared with the homologous lipid-water phase and that the protein content of the phase decreases as the ratio of the liquid to rigid hydrocarbon chains decreases. This leads us to suggest that in the systems studied in this work the proteins interact only with lipid in the liquid-like state. In the case of the phases with hydrophobic interaction, it is shown that the extent of hydrophobic interaction between protein and lipid increases as the unsaturation of the hydrocarbon chains increases. The onset of the order-disorder transition shows a greater shift towards low temperature than the one observed in the case of the phase with electrostatic interaction.

Introduction

Order-disorder conformational transition of the hydrocarbon chains of lipids, a well known phenomenon in lipid-water systems, has been shown to

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take place in intact membranes [1—4]. This observation has aroused considerable interest for this type of phenomenon and for its possible biological implications. We have described the phases involved in the conformation transition for a variety of lipid-water systems and the influence of various parameters (chemical heterogeneity of the chains, degree of hydration and nature of the polar groups) on the transition [5]. We have also established a relationship between the rapid motions of the lipid chains and the structure of some lipid-water and protein-lipid-water phases [6]. We extend here those observations to protein-lipid-water phases with a particular concern for the influence of the protein-lipid interactions on the order-disorder conformational transition.

According to previous studies of our laboratory [7,8] we distinguish in this work two types of protein-lipid interactions, which we call 'electrostatic' and 'hydrophobic'. The interactions of the 'electrostatic' type are characterized by the fact that the partial thickness (see definition below) of the lipid bilayer is the same in the presence and in the absence of proteins, and by the ease with which the proteins and the lipids can be dissociated by raising the ionic strength. Those of the 'hydrophobic' type are characterized by a decrease of the partial thickness of the lipid layer in the presence of proteins and by the lack of dissociation when ionic strength is raised. These concepts have been discussed elsewhere [7,8] and several arguments have been produced in support of the notion that in the lipoprotein phases with hydrophobic interactions some of the hydrocarbon chains of the lipids come into contact with the hydrophobic core of the proteins.

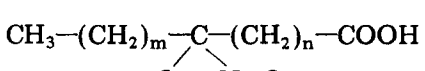
The systems used in this work involve either lysozyme or cytochrome *c* and several fractions of phosphatidic acid, each with a different content of unsaturated hydrocarbon chains.

Materials and Methods

Phosphatidic acid was prepared by enzymatic degradation of egg lecithin [9]. Different fractions were separated by step-wise crystallization from methanol solutions at different temperatures [9]. The fatty acid composition of the four fractions used in this work is shown in Table I; these fractions differ by the average degree of saturation and length of the hydrocarbon chains. The proteins were purchased from Sigma. Horse heart ferricytochrome *c* was used without further purification, hen egg lysozyme was dialyzed and freeze-dried before use. The procedures adopted for preparing the lipoprotein phases are described below (see *Phase analysis*).

The X-ray diffraction techniques are those commonly used in our laboratory.

The spin-labelled fatty acids I(*m*,*n*) were synthesized by the methods of Jones [10] and Keana et al. [11].



The molar label concentration was approximately 1/300. The EPR spectra were recorded on a Varian X-band spectrometer. The interpretation of the

TABLE I

FATTY ACID COMPOSITION OF THE FOUR FRACTIONS OF PHOSPHATIDIC ACID

Figures are % fractions.

Paraffin chains	Fractions			
	1	2	3	4
$C_{16:0}$	26.5	31.5	37.0	41.2
$C_{16:1}$	5.7	0.6	1.4	2.3
$C_{18:0}$	8.8	16.1	13.6	18.8
$C_{18:1}$	33.0	39.5	31.9	26.3
$C_{18:2}$	26.0	12.3	16.1	11.4

spectra has been given by McConnell et al. [12]. We used $T/\!/$ as a measure of the amplitude of the rapid reorientational motions of the nitroxide radical [6]. In all the experiments, the temperature of the samples was controlled within 1°C.

Phase analysis

The first step in the study of multi-component systems, which happens to be of critical importance in this work (see below), is to identify the different phases present in each preparation and to determine their chemical composition. For these purposes we adopted the following procedures.

The protein-lipid-water samples were prepared by adding a fixed volume of water dispersion of lipids (approx. 5 mg/ml) to a large excess of protein solution (approx. 15 mg/ml); under these conditions a precipitate is obtained which contains all the lipids interacting with the protein. This precipitate, after elimination of excess solvent by centrifugation, provides the sample used for the X-ray and the ESR experiments. The presence of only one phase in the sample is tested by two criteria. One is the observation of only one family of small-angle X-ray reflections which can all be indexed according to one lattice. The other is the optical homogeneity and transparency of the sample which is generally achieved within a very narrow range of water content. If water is added or withdrawn the preparation becomes turbid. The weight of lipids before precipitation, the amount of protein added and of protein remaining in the supernatant are determined in each experiment. A further chemical analysis of the protein, lipid and water content is carried out on the samples which according to the previous criteria appear to contain only one phase.

In the first run we performed the temperature-dependent X-ray and ESR experiments on samples obtained by precipitation at room temperature. The results showed that lowering the temperature sometimes altered the chemical composition of the phases, and thus increased the number of phases present in the preparation. In order to keep these parameters under control, some experiments were performed on samples obtained by precipitation at the temperature of the X-ray experiments.

X-Ray diffraction

Room temperature. In all the cases the precipitate obtained at room

temperature is optically homogeneous and transparent and yields an X-ray diffraction diagram with one family of small angle reflections, all integral orders of one fundamental repeat. Moreover, the presence of a diffuse band near $(4.6\ \text{Å})^{-1}$ shows that the conformation of the hydrocarbon chains is liquid-like (type α, ref. 13). These observations indicate that each system consists of a lamellar phase; therefore the composition of the phase can be determined by the chemical analysis of the sample. The structure of these phases may be visualized to consist of lipid bilayers with intercalated layers of protein and water (Fig. 1). The partial thickness of the protein, lipid and water layers (namely the thickness of the ideal planar slabs containing respectively all the protein, lipid and water of one unit cell) in the one-dimensional unit call can be determined when the repeat distance, concentration and partial specific volumes of the components are known [7]; the results are given in Table II.

The results in Table II deserve several comments. The partial thickness of the lipid layer in the phases prepared with lysozyme at pH 6 and with any of the four lipid fractions is very close to the thickness of the lipid bilayer in the lamellar phases of the lipid-water system. Furthermore the lipids are readily dissociated from the proteins even at low ionic strength (0.25 M NaCl). Therefore, in these phases the protein-lipid interactions are typically 'electrostatic'. We call these phases e_{lys}. On the contrary the phases prepared with lysozyme at pH 4 display protein-lipid interactions of the 'hydrophobic' type. Indeed, the lipid layer shrinks in the presence of protein and the dissociating effect of salt is negligible; moreover, it may be noted that the thickness of the lipid layer decreases as the degree of unsaturation of the chains increases. We call these phases h_{lys}.

The properties of the phases made with cytochrome *c* at pH 4 are intermediate. The lipid leaflet is thinner in the presence than in the absence of protein; the thinning effect increases and the dissociating effect of salts decreases as the average degree of saturation of the chains decreases. In all cases the thickness of the lipid layer, the dissociating effect of salt and the degree of

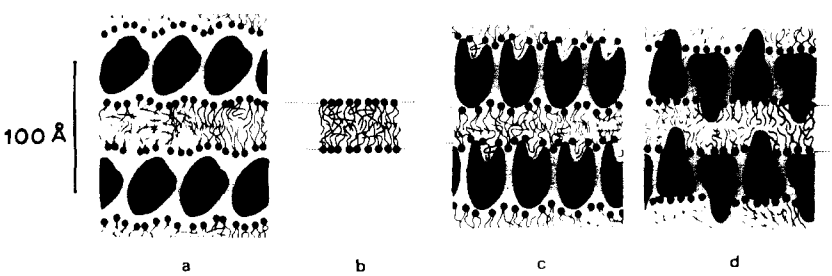


Fig. 1. Schematic representation of the structure of the protein-lipid-water lamellar phases of the 'electrostatic' and the 'hydrophobic' type. The sections are perpendicular to the plane of the lamellae. The protein molecules are represented by densely hatched objects of irregular shape, the polar groups of the lipids by black dots and the hydrocarbon chains in the α conformation by wriggles. The lipid-water phase is represented in b, the protein-lipid-water phase in a, c and d. a represents a structure with 'electrostatic' protein-lipid interactions: note that the thickness of the lipid leaflet is the same in this phase as in the lipid-water phase. c and d represent structures with 'hydrophobic' interactions: note that the partial thickness of the lipid layer is smaller than in a and b. In c the hydrophobic interaction involves hydrophobic chains penetrating the hydrophobic core of the protein molecules, in d the protein molecules protrude into the lipid layer. (By courtesy of Drs. D.M. Sadler and T. Gulik-Krzywicki).

TABLE II

CHEMICAL DATA AND DIMENSIONS RELEVANT TO SOME PHASES

The phases were prepared as described in the text. c_p, c_l and c_w are the weight concentrations of each component; d is the repeat distance; d_p, d_l and d_w are the 'partial thicknesses' of the protein, lipid and water layers in the one-dimensional unit cell (see text). The amount of protein dissociated from the lipids by the salt treatment was determined as follows: a weighed lipoprotein sample was incubated under agitation with a salt solution of known molarity for a few minutes, the suspension was centrifuged and the amount of released protein was estimated in the supernatant by absorption at 280 nm.

Phase	Lipid fraction	c_p	c_l	c_w	d(Å)	d_p	d_l	d_w	% Dissociation by NaCl solutions (M)		
									1	0.5	0.25
Phosphatidic	1	—	0.60	0.40	65.0	—	38.4	26.6	—	—	—
acid-water	2	—	0.56	0.44	71.0	—	39.1	31.9	—	—	—
(L_α)	3	—	0.68	0.32	58.4	—	39.2	19.2	—	—	—
	4	—	0.73	0.27	55.0	—	39.7	15.3	—	—	—
Lysozyme-	1	0.39	0.39	0.22	95.0	30.3	40.9	23.8	100	90	76
phosphatic acid,	2	0.39	0.39	0.22	95.3	30.6	40.7	24.0	100	96	76
pH 6 (e_{lys})	3	0.39	0.39	0.22	97.0	31.2	41.6	24.2	100	100	80
	4	0.39	0.39	0.22	97.6	31.2	41.2	25.2	—	—	—
Lysozyme-	1	0.36	0.33	0.31	73.4	21.4	25.8	26.2	0	0	0
phosphatic acid,	2	0.35	0.34	0.31	75.5	21.1	28.0	26.4	0	0	0
pH 4 (h_{lys})	3	0.33	0.37	0.30	76.3	20.4	30.1	25.8	0	0	0
	4	0.32	0.38	0.30	77.4	20.2	31.8	25.4	—	—	—
Cytochrome c-	1	0.33	0.44	0.23	74.3	19.8	35.3	19.2	32	28	24
phosphatic acid,	2	0.32	0.45	0.23	76.6	20.1	36.7	19.8	56	52	49
pH 4 (h_{cyt})	3	0.32	0.45	0.23	77.8	20.4	37.6	19.8	89	82	79
	4	—	—	—	—	—	—	—	—	—	—

saturation of the lipids display clear correlations, consistent with the notion that the extent of the hydrophobic contacts between lipids and proteins increases as the degree of unsaturation of the paraffin chains increases. We call these phases h_{cyt}.

Temperature-induced transitions

As the temperature is lowered, several phenomena occur; in describing these phenomena we may distinguish the 'high' and the 'small' angle regions of the diffraction diagram (the separation may be set at approximately $(7\ Å)^{-1}$) [5].

With regard to the high angles, at room temperature we observe only the $(4.6\ Å)^{-1}$ diffuse band. As the temperature is lowered, the $(4.2\ Å)^{-1}$ sharp reflection becomes visible at first and then its intensity increases at the expenses of that of the band. We have shown in the study of lipid-water systems how the ratio of the intensity of the sharp reflection to that of the diffuse band can be used to estimate the fractions of chains in the α and in the β conformations (respectively c_α and c_β), by comparing the microdensitometer tracings of the high-angle region with a weighted linear combination of the tracings obtained with lipid phases entirely in the α and in the β conformation [5].

In protein-lipid-water systems the situation is complicated by the presence of a diffuse band due to proteins in the region between $(5\ Å)^{-1}$ and $(4\ Å)^{-1}$. Under

these conditions the intensity of the band at $(4.6\ \text{Å})^{-1}$, and therefore the content of the chains in the α conformation, tend to be overestimated; nevertheless, since the protein content is almost the same this procedure applied to the three lipoprotein phases provides an indication of the relative amount of chains in the α and in the β conformations. Fig. 2 shows a few examples of this treatment of the high angle region; the values of c_β so obtained are plotted as a function of temperature in Fig. 3. As may be noted, the onset of the $\alpha \rightarrow \beta$ transition and the fraction of chains in the β conformation are different in the three cases and depend on the nature of the protein-lipid interactions. It is worth noting that this estimate of the fraction of the chains in the β conformation is, to a large extent, independent of the number of phases present in the system.

With regard to the 'small' angles, the experimental observations are different in the different phases; we discuss these observations below.

Phases of the electrostatic type. In this type of phase the onset of the $\alpha \rightarrow \beta$ transition is shifted to lower temperatures by a few degrees with respect to the corresponding lipid-water phases (see Fig. 3). Furthermore, at the lowest temperature which can be reached without freezing the water, and thus disrupting the protein-lipid-water phase, the fraction of the chains in the β con-

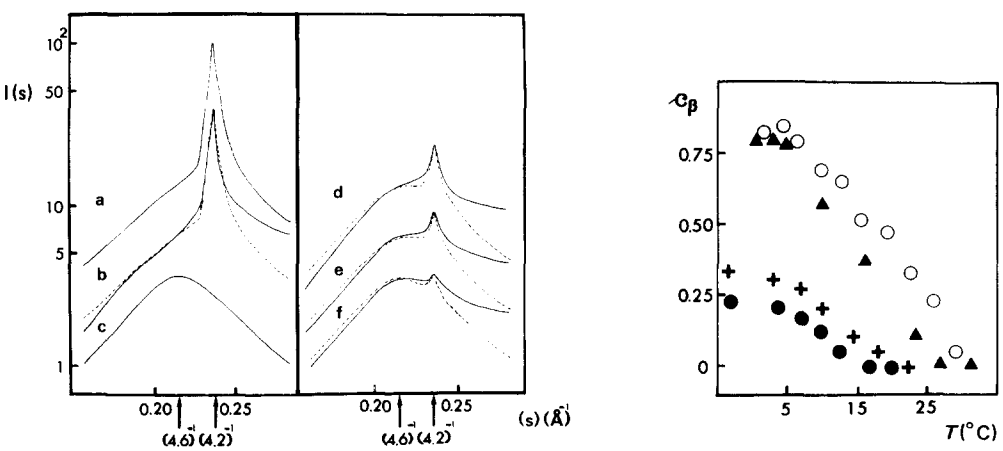


Fig. 2. Microdensitometer tracings of the high angle region relevant to phases prepared with the lipid fraction 3 (see Table I). Solid lines: the curves a and c are those of the phases L_β and L_α of a sample of egg-lecithin ($c = 0.94$) which yields L_β practically pure at 0°C (curve a) and L_α pure at 40°C (curve c); the two X-ray diffraction experiments were performed on the same sample with identical exposure times. Curve b corresponds to the lipoprotein phase e_{lys} at 3°C; the low temperature phase was pure at this temperature. Curves d, e and f are experimental curves corresponding to the lipoprotein phase h_{lys} (hydrophobic type) at —10, 8 and 12°C respectively. Dotted lines: the dotted curves in the three frames are weighted linear combinations of the continuous curves a and c (left frame) in the proportions which gives the best agreement with the experimental curves. The values of c_β so obtained are shown in Fig. 3. The poor agreement of the curves at $s > (4\ \text{Å})^{-1}$ is explained by the water content, which is higher in the lipoprotein phases than in the egg lecithin sample (see ref. 5).

Fig. 3. Fraction c_β of chains in the β conformation, estimated according to the intensity distribution in the high angle region (see text and Fig. 2). The protein-lipid-water phases were obtained with fraction 3 (see Table I). The results obtained with the phase $L_{\alpha\beta}$ of phosphatidic acid (○) are given as reference [5]. Temperature-dependent X-ray experiments were performed on samples obtained by precipitation at 30°C. At 30°C each sample contains only one phase: ▲, e_{lys}; +, h_{cyt}; ●, h_{lys}.

formation is similar in the presence and in the absence of protein ($c_\beta \simeq 0.8$, see Fig. 3).

In the small angle region two families of sharp lamellar reflections are observed throughout the transition: a high temperature one with repeat distance 97 Å, a low temperature one with repeat distance 92 Å. In the case of the phase made with the lipid fraction 3 the onset of the transition is at 24°C; between 24°C and 3°C the two phases are present, the intensity of the reflections of the low temperature phase increasing as the temperature is lowered. The relfections of the high temperature phase vanish near 3°C. Moreover, the repeat distance, the relative intensity of the small angle reflections and the ratio of the intensity of the $(4.2\ Å)^{-1}$ to that of the small angle reflections are independent of temperature. It can thus be inferred that the structure of the low temperature phase is the same at all temperatures, and that the effect of temperature is to alter the relative amount of the high and the low temperature phases.

In order to pursue the analysis of the low temperature phase its composition must be determined. Fig. 4 shows that in the samples precipitated at low temperature the protein/lipid ratio decreases with lowering temperature. A complete chemical analysis was performed on the samples precipitated at 30°C and at 3°C, which contain only one phase (see above). The results are $c_p = 0.39$, $c_l = 0.39$. $c_w = 0.22$ at 30°C; $c_p = 0.17$, $c_l = 0.53$, $c_w = 0.30$ at 3°C. The partial thickness of the protein, lipid and water layers are: $d_p = 31.2$ Å, $d_l = 41.6$, $d_w = 24.2$ Å at 30°C; $d_p = 12.3$ Å, $d_l = 50.4$ Å, $d_w = 29.3$ Å at 3°C. The intensity of a sharp reflection at $(4.2\ Å)^{-1}$ indicates that in the low temperature phase most of the chains are in the β conformation; the amount can be estimated to be approx. 0.8 according to the relative intensity of the $(4.2\ Å)^{-1}$ reflection to that of the $(4.6\ Å)^{-1}$ band (see Fig. 3). Assuming by analogy with the phase $L_{\alpha\beta}$ of phosphatidic acid [5] that each lipid lamella of the low temperature phase is a mosaic of domain in the α and in the β conformation, the fraction of the chains in the β conformation can be estimated independently

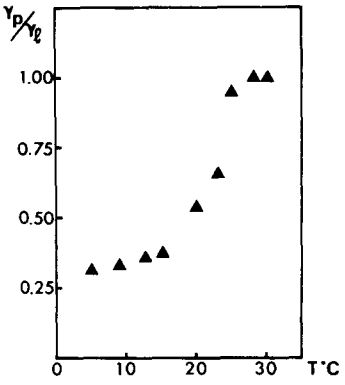


Fig. 4. Chemical composition of the phase e_{lys} as a function of the temperature at which centrifugation is performed (see text). γ_p is the weight difference between total protein and the protein remaining in the supernatant (see text). γ_l is the total weight of lipid before precipitation. According to this operational definition, γ_p is the weight of protein in the sample; the weight of lipid in the sample may be smaller than γ_l if some of the lipids do not interact with the protein and remain in the supernatant.

by comparing the partial thickness of the lipid layer with that of the phases L_α and L_β of the same lipid, in the absence of proteins [5]. The result is $c_\beta = 0.78$, in very close agreement with the previous estimate. For the low temperature phase we are thus led to put forward a structure which consists of stacked lipid bilayers, each of which may be visualized as a disordered patchwork of domains in the α and in the β conformation, with the protein molecules sparcely distributed in between (see Fig. 5). Moreover, the fact that the repeat distance of the low temperature phase is so close to that of the high temperature one (92 and 97 Å, respectively) suggests that the protein molecules play the role of spacers, in contact only with the lipids in the α conformation; thus the lipids with stiff chains appear to reject the protein, and the protein-lipid interactions are similar in the two phases.

A further element which suggests this structure is provided by the electron density profiles (see Fig. 6 and ref. 5). Indeed, the profile of the low temperature phase turns out to be quite similar to an average of the profile of the high temperature phase (with weight 0.2) and of the profile of the phase L_β of phosphatidic acid (with weight 0.8) [5].

Phases of the hydrophobic type. As the temperature is lowered a conformational transition of the hydrocarbon chains is observed. The onset of the $\alpha \rightarrow \beta$ transition is shifted to a lower temperature with respect to the lipid-water phase and the fraction of chains in the β conformation is smaller than in lipid-water and in the protein-lipid-water phases of the 'electrostatic' type (Fig. 3). Moreover, c_β is found to decrease as the extent of the 'hydrophobic' interactions increases (not shown in the figure).

One predominant family of lamellar small-angle reflections is observed in the temperature range 3—30°C; the spacings and relative intensity of these reflections remain constant. In addition to these reflections one sharp and faint reflection at $s = (17\ \text{Å})^{-1}$ is observed in the cases in which the intensity of the $(4.2\ \text{Å})^{-1}$ reflection, and thus the number of chains in the β conformation, is the highest. Therefore, it appears that the temperature-induced transition is similar to that observed in the phases of the 'electrostatic' type and involves

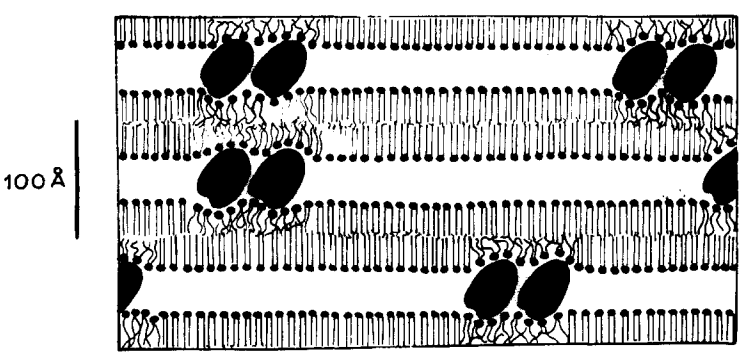


Fig. 5. Schematic view of the structure of the low temperature protein-lipid-water phase, e_{lys}. The straight lines represent the paraffin chains in the β conformation; see also legend of Fig. 1. This phase consists of stacked lipid lamellae, each of which is a random mosaic of domains in the α and in the β conformation, with protein molecules sparcely distributed between the bilayers. The proteins are associated only with the lipids whose chains are in the α conformation (see text).

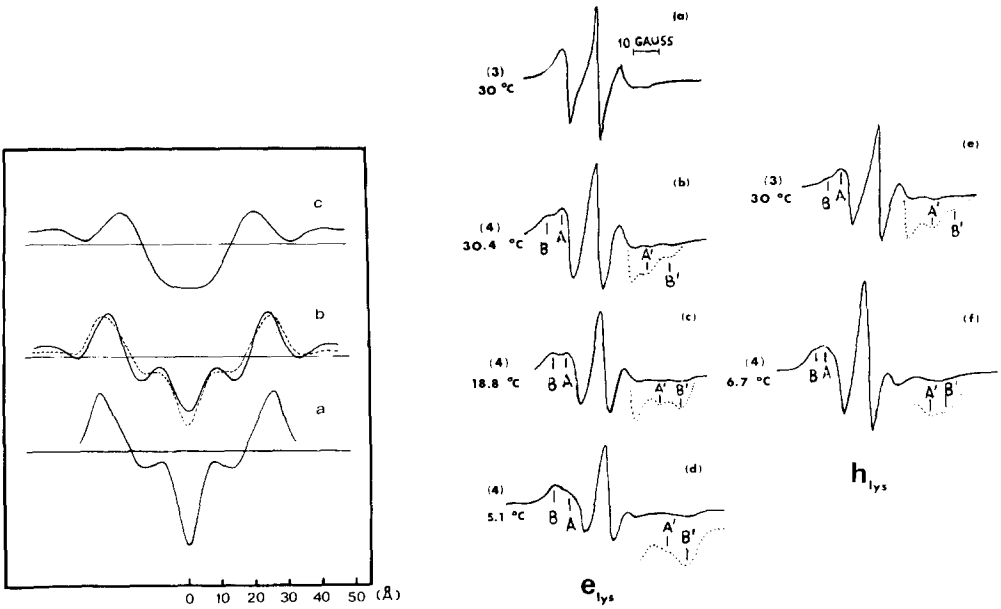


Fig. 6. Electron density profiles. Solid lines, experimental profiles; dotted line, linear combination of curves a and c, with weight 0.8 and 0.2 respectively (see text). a, Phase L_β of phosphatidic acid [5]; b, low temperature phase e_{lys} ($F(1) = +2.27$; $F(2) = -2.02$; $F(3) = 0$; $F(4) = +0.94$; $F(5) = +1.18$; $F(6) = -1.24$); c, high temperature phase e_{lys} [$F(1) = +1.45$; $F(2) = -1.47$; $F(3) = +0.97$; $F(4) = 0$; $F(5) = -0.67$]. The amplitudes of the structure factors were observed; the signs chosen are those which lead to the Fourier transforms which agree the best with the models adopted according to the criteria discussed in the text.

Fig. 7. Typical ESR spectra of the label I(5,10) incorporated in the phases e_{lys} and h_{lys}. The dashed lines represent part of the spectra on an expanded scale. The lipid fraction (in parenthesis, see Tables I and II) and the temperature are indicated for each spectrum. The signals [A, A'] and [B, B'] correspond to labels of high and low mobility, respectively. e_{lys}: at 30°C all the labels belong to the mobile species for the less saturated lipid (3), whereas some are immobilized for the more saturated lipid (4); moreover, as the temperature is lowered the relative intensity of the low mobility signal increases. h_{lys}: the two signals indicate that two classes of label are present, one with a low, the other with a high mobility.

two phases, one with all the chains in the α, the other with a fraction of the chains in the β conformation. Yet the concentration of the low temperature phase is never sufficiently high for a determination of its composition and structure.

Electron spin resonance

The phases e_{lys} and h_{lys} have been studied by X-ray diffraction and ESR techniques at room temperature [6]. The results can be briefly summarized as follows. In the phase e_{lys} the three labels I(13,2), I(10,5) and I(5,10) display single spectra with mobilities similar to those observed in the phase L_α of phosphatidic acid (Fig. 7a). In the phase h_{lys} the labels I(13,2) and I(10,5) also display single spectra with mobilities slightly smaller than those observed in the phase e_{lys}; the spectrum obtained with the label I(5,10) is the superposition of two spectra, and suggests that some of the labelled molecules are in contact with the protein (low mobility) and some with the lipid (high mobility) (see

Fig. 7e). These results are barely dependent on the fraction of phosphatidic acid used.

Phases of the electrostatic type. As the $\alpha \rightarrow \beta$ transition proceeds, the spectra observed with all the labels are the superposition of two distinct components corresponding to high and low mobility species, respectively, and the relative intensity of the low mobility component increases as the temperature is lowered (Fig. 7, b, c and d). As it may be expected from the 'fluidity gradient' across a lipid monolayer [6,14,15]. The difference between the two values of $T/\!/$ decreases with increasing distance of the oxazolidine ring to the polar end of the label (Fig. 8). Furthermore, varying the degree of saturation of the chains has the effect of shifting the temperature of the transitions (see Fig. 8).

At all temperatures within the transition range the spectra are the superposition of two components, the intensity of the low mobility signal increasing with lowering temperature (at very low temperature the high mobility signal becomes so weak that $T/\!/$ cannot be measured, see Fig. 7). Moreover, even at the lowest temperature the spectra do not show any indication of dipolar interactions between the labels; since at low temperature 80% of the chains are in the β conformation, this observation indicates that the three labels are soluble in the two phases and in the β as well as in the α domains. In the phase $I_{\alpha\beta}$ of phosphatidic acid we found that in the course of the $\alpha \rightarrow \beta$ transition the labels I(5,10) and I(10,5) are rejected out of the domains in the β conformation [6]. Therefore, it appears that the presence of proteins shifts the partition of this label between the α and the β regions in favour of the latter. This conclusion is in agreement with the conclusion of the X-ray study that the low temperature phase contains some protein (see above and Fig. 5).

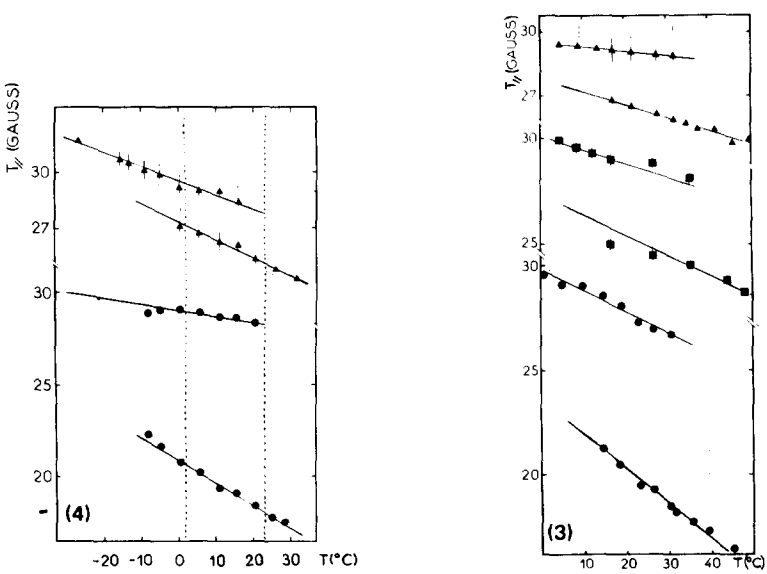


Fig. 8. Temperature dependence of the hyperfine splitting $T/\!/$ of the labels I(13,2) (▲), I(10,5) (■) and I(5,10) (●) incorporated in the phase e_{lys}. Figure in parenthesis characterizes the lipid fraction. Between the dashed lines the high and low temperature phases are both present (as shown by the X-ray study). The high mobility signal is still present at the lowest temperature (see Fig. 7), although it is so weak its position cannot be determined.

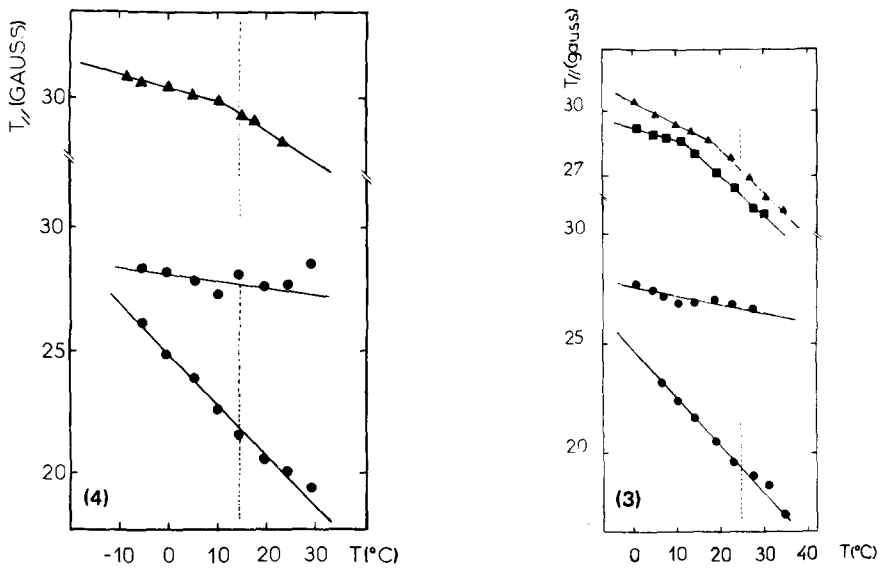


Fig. 9. Phase h_{lys}: notation as in Fig. 8. Note that only the label I(5,10) (•) yields a two-signal spectrum.

Phases of the hydrophobic type. In this type of phase the spectra of the labels I(13,2) and I(10,5) contain one component at all temperatures. Lowering the temperature decreases mobility and produces a kink in the curves *T//* (see Fig. 9). At high temperature the spectra of the label I(5,10) contain two components (see Fig. 7e). This observation is consistent with our previous interpretation [6], that some of the label is embedded in the hydrocarbon regions and some is in contact with the protein. In lowering the temperature the intensity of the low mobility signal increases with respect to the high mobility signal (Fig. 7f). The comparison of the low temperature spectra (6°C) obtained with h_{lys} and e_{lys} shows that the relative proportion of the immobilized component is smaller in h_{lys} than in e_{lys} (compare d and f in Fig. 7) as it is expected from the X-ray diffraction studies (see above). We have failed to find three component spectra at low temperature. Indeed, the *T//* values of the I(5,10) label in β domains and in contact with the proteins would be too close to produce a resolved spectrum.

Discussion

The most interesting conclusion of this work is that in the two types of lysozyme-phosphatidic acid-water phases studied (one with electrostatic, the other with hydrophobic protein-lipid interactions) the protein molecules are in contact only with the lipid molecules whose chains are in α conformation; the lipid domains in the β conformation are devoid of protein. This conclusion can be compared with the conclusion of recent studies on intact membranes and extracted membrane proteins [16—19] where it has been shown that membrane proteins are excluded from β domains.

Our results confirm the validity of the operational criterias (partial thickness of the lipid layer, dissociating effect of salt, spectroscopic properties) on which

we have previously founded a distinction of the protein-lipid interactions in the two main types, electrostatic and hydrophobic [4,5]. In fact, the system cytochrome *c*-phosphatidic acid-water provides an example of gradual transition between the two types of interactions (see Table II). This work also confirms previous observations that the unsaturation of the chains promotes the interactions of the hydrophobic type [8] and that in the phase h_{lys} the label I(5,10) segregates between two sites, one apparently embedded in the lipid matrix the other in contact with the protein [6].

Another result which extends our previous observations [5] is that the protein-lipid interactions have a strong influence on the temperature-induced conformational transitions of the hydrocarbon chains. In the presence of proteins the $\alpha \rightarrow \beta$ transition is shifted to lower temperature: this effect is stronger if the lipid-protein interactions are of the 'hydrophobic' type. In the case of the lysozyme-lipid phase of the 'electrostatic' type the conformational transition involves two phases. In the high temperature phase all the chains are in the α conformation (Fig. 1a). The low temperature phase consists of stacked lipid lamellae, each a random mosaic of domains in the α and in the β conformation, with proteins sparcely distributed in between (Fig. 5). The presence of fairly large amounts of water intercalated between the lipid domains in the β conformation is probably related to the electrical charges of the lipids [5]. The conformational transition of the phase with 'hydrophobic' interactions appears to be essentially similar to that of the phase with 'electrostatic' interactions, although this point is not fully explored in this work.

It should be noted that the ESR experiments suggest that in the low temperature phase of e_{lys} the structure of the α and of the β domains is not quite the same as in the phase $L_{\alpha\beta}$ of phosphatidic acid [5]. Indeed, in the phase $L_{\alpha\beta}$ all the labels appear to be rejected by the β domains, whereas in the presence of lysozyme the labels are distributed in the two types of domains.

With regard to ESR, our experimental results, which at first sight may look rather complicated, are in fact easily explained once the structure of the phases involved is known. It is worthwhile to stress once more [6] how sensitive the ESR spectra are to the nature of the label, and how difficult it would be to draw conclusions without taking into account the specific properties of the labels used and without knowing the structure of the system.

A few final comments of more general interest: previous experience in the X-ray diffraction study of membranes and model systems has led us to emphasize the importance of the chemical data (composition, partial specific volumes) associated to lattice symmetry and dimensions, and to question the usefulness of electron density profiles [20]. This paper provides additional illustration of that conclusion; the correct interpretation of the low temperature X-ray and ESR experiments is critically dependent upon the chemical composition of the phases, which had to be determined by performing the chemical and physical experiments at the same temperature. A second point which has often been raised in the past is that the interpretation of the spectroscopic experiments, and more generally the reliability of these techniques for the study of membranes, hinges upon a precise structural information, or at least requires a careful calibration on systems of known structure; this point also is illustrated by the results of the ESR study reported here. More specifically, it may be

questioned to what extent the ESR spectra of labels incorporated in biological membranes are likely to provide information on structure and structural transitions, especially when the conformational transitions involved are likely to entail complex segregation phenomena.

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