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THE POLYMORPHIC PHASE BEHAVIOUR OF PHOSPHATIDYLETHANOLAMINES OF NATURAL AND SYNTHETIC ORIGIN

A ^{31}P NMR STUDY

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Summary

1. The polymorphic phase behaviour of aqueous dispersions of phosphatidylethanolamines isolated from human erythrocytes, hen egg yolk and *Escherichia coli* have been investigated employing ^{31}P NMR techniques. All species exhibit well defined, reversible bilayer to hexagonal (H_{11}) phase transitions as the temperature is increased. The temperatures at which these transitions take place (10, 25–30 and 55–60°C for erythrocyte, egg yolk and *E. coli* phosphatidylethanolamine, respectively) are sensitive to the fatty acid composition, occurring at a temperature up to 10°C above the high temperature end of the hydrocarbon phase transition as detected by differential scanning calorimetry. In some cases the bilayer to hexagonal (H_{11}) transitions may also be detected employing calorimetric techniques.

2. The addition of equimolar concentrations of cholesterol to these naturally occurring phosphatidylethanolamines does not dramatically affect the bilayer-hexagonal (H_{11}) transition temperature, producing changes of up to 10°C.

3. 18 : 1_t/18 : 1_t phosphatidylethanolamine undergoes the bilayer to hexagonal (H_{11}) phase transition as the temperature is increased through the interval 50–55°C. Alternatively, hydrated 12 : 0/12 : 0 phosphatidylethanolamine remains in the bilayer phase at temperatures up to 90°C (50°C above the hydrocarbon phase transition temperature).

4. The presence of 100 mM NaCl or 10 mM CaCl₂ in aqueous dispersions of egg yolk phosphatidylethanolamine does not alter the temperature-dependent polymorphic phase behaviour significantly. However, at 40°C, increasing the

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p^2H above 8.0 results in progressive inhibition of the hexagonal (H_{11}) phase and the appearance of a phase possibly of cubic structure at p^2H 9.0. At p^2H 10.0 the bilayer phase is preferred.

5. It is suggested that in biomembranes containing phosphatidylethanolamine as a majority species (such as that of *E. coli*) the fatty acid composition may primarily reflect the need to maintain bilayer structure. Alternatively, it is pointed out that in mammalian membranes such as that of the erythrocyte, phosphatidylethanolamine tends to destabilize bilayer structure. The resulting possibility that transitory non-bilayer lipid configurations may occur may be directly related to many important properties of biological membranes.

Introduction

Phosphatidylethanolamines are a major constituent of most mammalian and bacterial cell membranes. It is therefore remarkable that the physical properties of phosphatidylethanolamines and their relation to the structure and function of biological membranes has received little attention. It has been known for some time that phosphatidylethanolamines from natural sources [1–3] often assume non-bilayer configurations such as the hexagonal (H_{11}) phase at physiological temperatures and pH values, but the influence of such details as fatty acid composition, ionic strength as well as the possible effects of high concentrations of ‘non-bilayer’ lipid in biological membranes has been largely ignored. More recently, however, it has been shown that unsaturated synthetic [4] and naturally occurring [5] phosphatidylethanolamines undergo bilayer to hexagonal (H_{11}) phase transitions at temperatures which appear sensitive to the fatty acid composition. Further, it has been pointed out that non-bilayer phases such as the hexagonal (H_{11}) phase may be intimately involved in such processes as transbilayer ‘flip-flop’ [5] and membrane fusion [6] phenomena.

In this context it is clearly important to characterize the physical properties of phosphatidylethanolamines, with particular emphasis on the polymorphic phase behaviour and factors which may influence it, as a first step in understanding the roles they may play in biological membranes. Such studies are described here for phosphatidylethanolamines isolated from the (human) erythrocyte, (hen) egg yolk and *Escherichia coli* as well as two species of phosphatidylethanolamine of synthetic origin. ^{31}P NMR techniques are employed which, as indicated elsewhere [5], provide a most convenient method for investigating the polymorphic phase behaviour of phospholipids in model [4,5, 7,8] as well as biological [6,9] membranes.

Materials and Methods

Phosphatidylethanolamine was isolated from the total lipids of human erythrocytes, egg yolk and *E. coli* employing silicic acid column chromatography followed by carboxymethyl-cellulose column chromatography [10], resulting in phosphatidylethanolamine that was at least 99% pure as indicated by thin-layer chromatography. 1,2-Dilauroyl-*sn*-glycero-3-phosphorylethanolamine (12 : 0/12 : 0 phosphatidylethanolamine) and 1,2-dielaidoyl-*sn*-glycero-

3-phosphorylethanolamine ($18 : 1_t/18 : 1_t$ phosphatidylethanolamine) were synthesized as described previously [4]. Cholesterol was obtained from Fluka (Buchs, Switzerland).

Hydrated dispersions of lipid were obtained from 50 to 100 mg phospholipid dissolved in chloroform, where the chloroform was removed by evaporation under nitrogen and subsequent overnight storage under vacuum. The lipid was then hydrated in 0.7 ml $^2\text{H}_2\text{O}$ (2 mM EDTA/25 mM Tris-acetic acid, p^2H 7.0) by exhaustive (up to 0.5 h) vortexing employing a glass agitator in the lipid dispersions. The lipid dispersion formed was a particulate precipitate.

^{31}P NMR spectra were obtained on a Bruker WH-90 Fourier Transform spectrometer operating at 36.4 MHz. All spectra were obtained in the presence of (20 W) broad band proton decoupling. Accumulated free induction decays were obtained from up to 10 000 transients, employing a delay time of 83 μs , an interpulse time of 0.17 s and a 45° radio frequency pulse.

Differential scanning calorimetry experiments were performed as described elsewhere [11].

Results

The ^{31}P NMR spectra obtained as a function of increasing temperature for erythrocyte phosphatidylethanolamine, egg phosphatidylethanolamine and *E. coli* phosphatidylethanolamine are illustrated in Fig. 1a, Fig. 1b and Fig. 1c, respectively. Employing previous identifications of the ^{31}P NMR lineshape with the polymorphic phase adopted by the phospholipid [4–8] these results indicate a bilayer to hexagonal (H_{11}) phase transition for erythrocyte phosphatidylethanolamine in the region of 10°C , for egg phosphatidylethanolamine in the region of $25\text{--}30^\circ\text{C}$ and for *E. coli* phosphatidylethanolamine in the region of $55\text{--}65^\circ\text{C}$. All these transitions were fully reversible with a hysteresis of no more than 5°C .

The calorimetric properties of aqueous dispersions of these three naturally occurring phosphatidylethanolamines are illustrated in Fig. 2. It may be noted that the bilayer to hexagonal (H_{11}) transition is observable for egg and erythrocyte phosphatidylethanolamine, particularly in the cooling scans. The bilayer to hexagonal transition is not resolvable employing calorimetric techniques for *E. coli* phosphatidylethanolamine however as the hydrocarbon phase transition occurs at the same temperature.

The observation of the bilayer-hexagonal (H_{11}) transition by calorimetric techniques for these naturally occurring phosphatidylethanolamines is in strong contrast to the behaviour observed for $18 : 1_c/18 : 1_c$ phosphatidylethanolamine [11], for which no calorimetric transition is observed. This disparate behaviour must be related to the very heterogeneous fatty acid composition of the natural phosphatidylethanolamines (cf. Table I) and indicates increased disorder of the acyl chains in the hexagonal (H_{11}) phase.

The fatty acid composition of erythrocyte, egg and *E. coli* phosphatidylethanolamine are given in Table I. The high hydrocarbon transition temperature of the *E. coli* phosphatidylethanolamine employed in this study is seen to result from the large proportion of cyclopropanecarboxylic fatty acids present, due to harvesting in the late exponential phase [12]. The increasing saturation

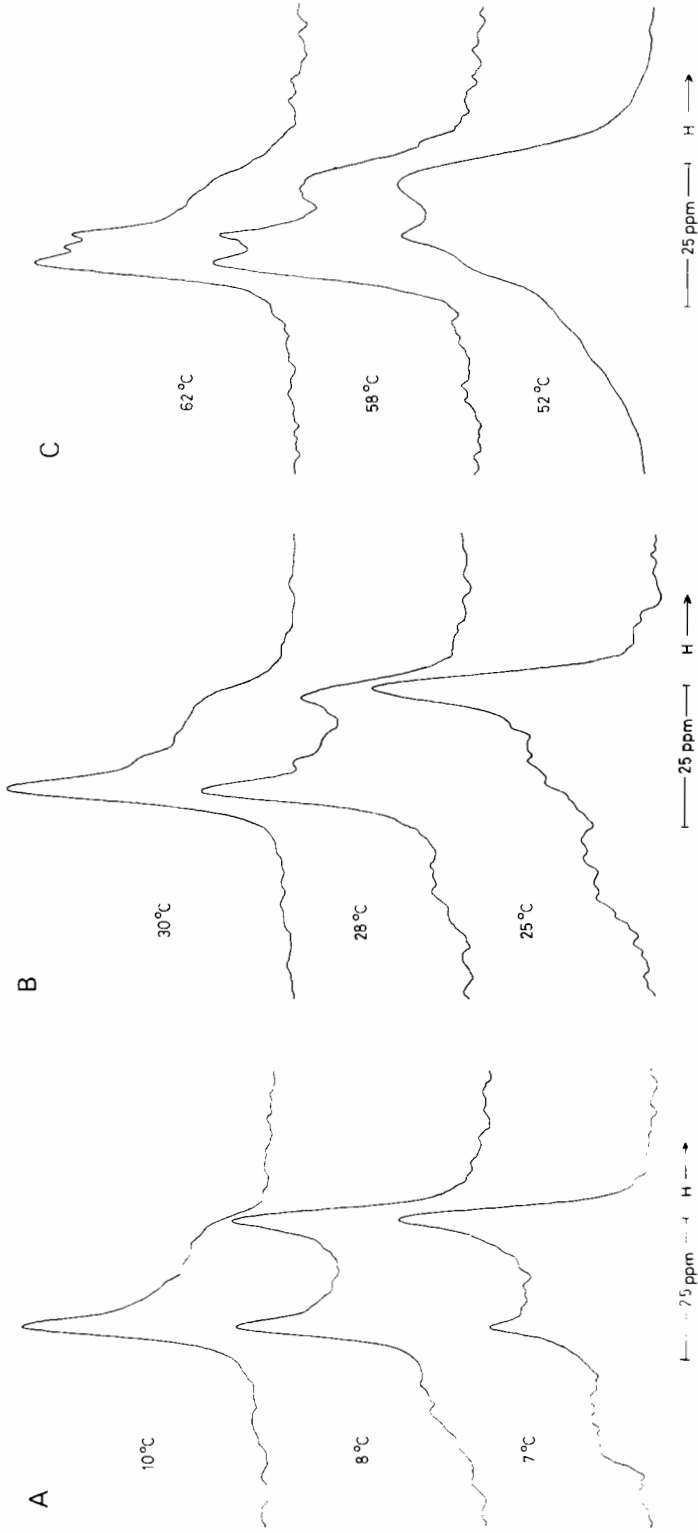


Fig. 1. 36.4 MHz, ^{31}P NMR spectra of aqueous dispersion of (a) human erythrocyte phosphatidylethanolamine; (b) hen egg yolk phosphatidylethanolamine; (c) *E. coli* phosphatidylethanolamine. All dispersions contained 25 mM Tris-acetic acid (p^2H 7.0) and 2 mM EDTA.

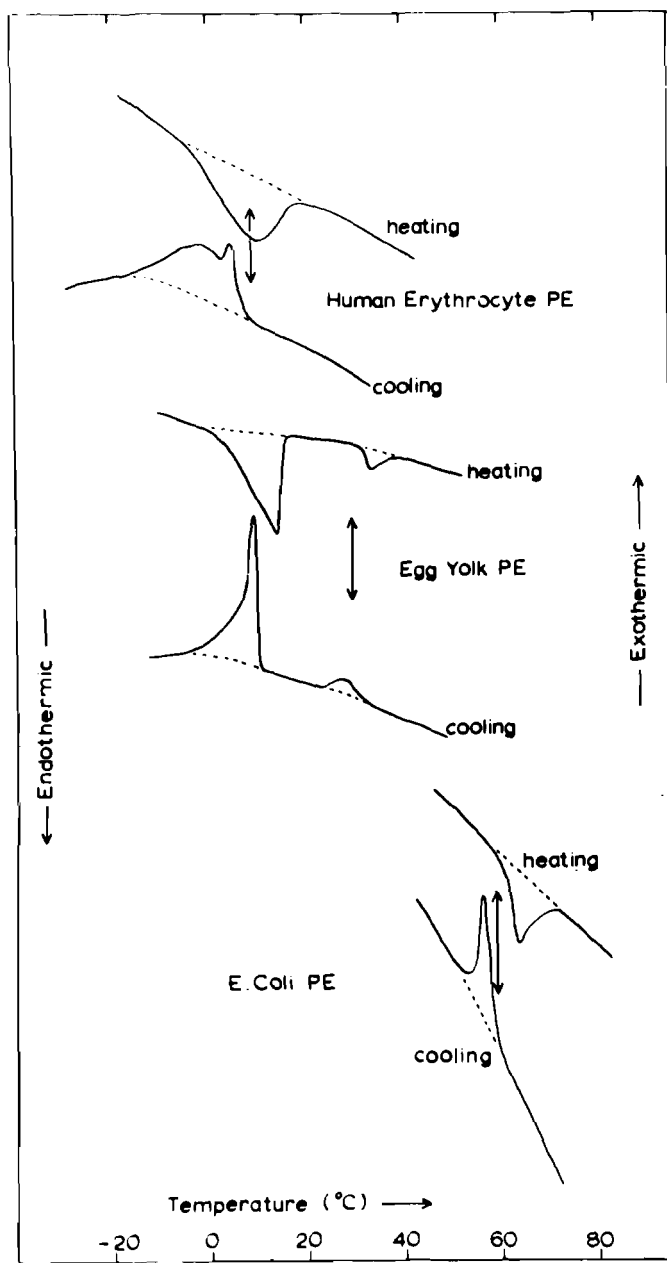


Fig. 2. Calorimetric scans of aqueous dispersions of human erythrocyte phosphatidylethanolamine, egg yolk phosphatidylethanolamine and *E. coli* phosphatidylethanolamine. A heating and cooling rate of $5^{\circ}\text{C}/\text{min}$ was employed. All dispersions contained 25 mM Tris-acetic acid (p^2H 7.0) and 2 mM EDTA. The erythrocyte phosphatidylethanolamine preparation also contained 30% by wt. ethylene glycol. The double-headed arrows indicate the midpoint of the bilayer-hexagonal (H_{11}) polymorphic phase transition as detected by ^{31}P NMR (see Fig. 4). PE, phosphatidylethanolamine.

TABLE I
 FATTY ACID COMPOSITION OF ERYTHROCYTE, EGG AND *E. COLI* PHOSPHATIDYLETHANOLAMINE

Species of phosphatidylethanolamine	Fatty acid (wt.%)															
	14:0	16:0	18:0	18:1	18:2	20:0	20:0	21:0	20:3	20:4	22:4	22:5	22:6	17:0 cyclo	19:0 cyclo	Unknown
Erythrocyte	—	18.7	12.6	21.9	6.9	0.5	—	—	1.2	18.7	4.7	2.7	3.5	—	—	8.7
Egg	—	17.7	30.2	17.7	9.6	—	—	—	—	15.9	1.9	—	6.9	—	—	—
<i>E. coli</i>	2.8	38.2	—	2.7	3.8	—	3.6	—	—	—	—	—	—	23.5	25.3	—

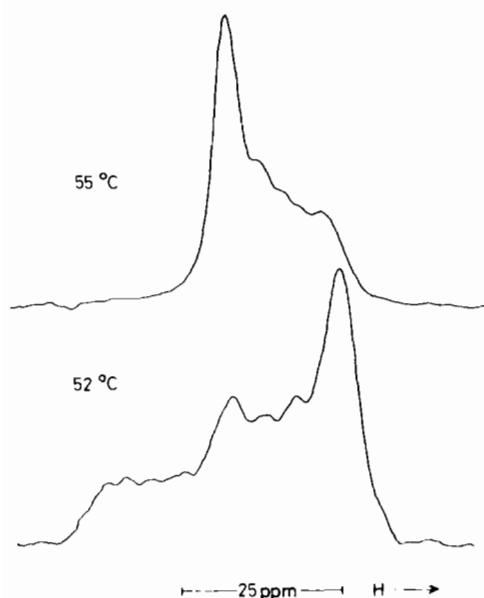


Fig. 3. 36.4 MHz ^{31}P NMR spectra of aqueous dispersions of 18 : 1_t/18 : 1_t phosphatidylethanolamine. The dispersion contained 25 mM Tris-acetic acid (p²H 7.0) and 2 mM EDTA.

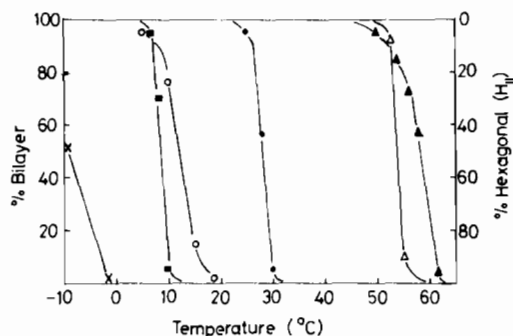


Fig. 4. Temperature-dependent polymorphic phase behaviour of various species of phosphatidylethanolamine of natural and synthetic origin. X, soya bean phosphatidylethanolamine; □, human erythrocyte phosphatidylethanolamine; ○, 18 : 1_c/18 : 1_c phosphatidylethanolamine; ●, hen egg yolk phosphatidylethanolamine; △, 18 : 1_t/18 : 1_t phosphatidylethanolamine; ▲, *E. coli* phosphatidylethanolamine. The fraction of lipid in the bilayer or hexagonal (H_{11}) phase was estimated by cutting and weighing the ^{31}P NMR spectra.

observed for erythrocyte, egg and *E. coli* phosphatidylethanolamine in turn corresponds to the progressive increase in the hydrocarbon transition temperatures as shown in Fig. 2.

In order to ascertain whether the ability of phosphatidylethanolamine to enter the hexagonal (H_{11}) phase is dependent on the presence of *cis*-unsaturated fatty acids or branched chain fatty acids, the polymorphic phase behaviour of 18 : 1_t/18 : 1_t phosphatidylethanolamine and 12 : 0/12 : 0 phosphatidylethanolamine was also investigated. As indicated in Fig. 3 18 : 1_t/18 : 1_t phosphatidylethanolamine enters the hexagonal (H_{11}) phase in the interval 50–55°C, whereas 12 : 0/12 : 0 phosphatidylethanolamine (data not shown) remains in the bilayer phase at temperatures as high as 80°C (50°C above its hydrocarbon phase transition temperature as detected by differential scanning calorimetry [11]).

The temperature-dependent polymorphic phase behaviour of the various species of phosphatidylethanolamine investigated here and in previous studies [4,5] are summarized in Fig. 4. It is to be noted that the temperature interval over which the bilayer to hexagonal (H_{11}) phase transition occurs is of the same order for phosphatidylethanolamines from natural sources, which have a very heterogeneous fatty acid composition, as it is for synthetic phosphatidylethanolamines with a single species of fatty acid components. This is in some contrast to the hydrocarbon phase transition which is markedly broader for

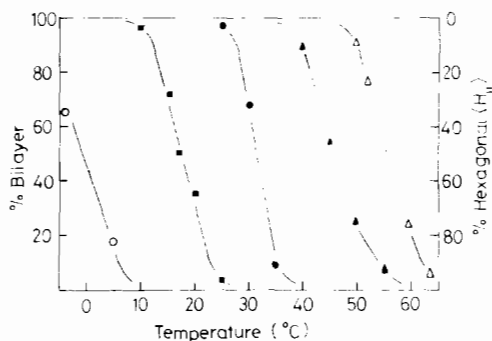


Fig. 5. Temperature-dependent polymorphic phase behaviour of various species of phosphatidylethanolamine in the presence of equimolar concentrations of cholesterol: \circ , 18 : 1_c/18 : 1_c phosphatidylethanolamine; \blacksquare , human erythrocyte phosphatidylethanolamine; \bullet , egg yolk phosphatidylethanolamine; \blacktriangle , *E. coli* phosphatidylethanolamine; \triangle , 18 : 1_t/18 : 1_t phosphatidylethanolamine. The amount of lipid in the bilayer or hexagonal (H_{11}) phase was estimated by cutting and weighing the various components of the ^{31}P NMR spectra.

phospholipids from natural sources (see, for example, ref. 13) than for well-defined synthetic phospholipids [14].

In Fig. 5 we summarize the temperature-dependent polymorphic phase behaviour of unsaturated phosphatidylethanolamines in the presence of equimolar concentrations of cholesterol. On comparing Fig. 5 with Fig. 4 it may be observed that cholesterol appears to have a different influence on the bilayer to hexagonal phase transition temperature depending on the fatty acid composition. In the case of 18 : 1_c/18 : 1_c phosphatidylethanolamine and *E. coli* phosphatidylethanolamine the transition temperature T_{BH} is lowered by approx. 10°C. Alternatively, T_{BH} is either not significantly affected or slightly higher for 18 : 1_t/18 : 1_t, egg and erythrocyte phosphatidylethanolamine. These results are very difficult to interpret for the naturally occurring phospholipids, as cholesterol may preferentially associate with particular molecular species present. That such possibilities may produce diverse effects is illustrated by the behaviour of mixed phosphatidylethanolamine-phosphatidylcholine systems, where the presence of cholesterol destabilizes bilayer structure when unsaturated phosphatidylcholines are present, but stabilizes bilayer structure when saturated phosphatidylcholines are introduced [5,7]. It is also possible that not all of the cholesterol introduced is contained in the phospholipid matrix. The clearest indication of the influence of cholesterol on the behaviour of different species of phosphatidylethanolamine is therefore available from the data obtained for 18 : 1_c/18 : 1_c and 18 : 1_t/18 : 1_t phosphatidylethanolamine, which would suggest that cholesterol lowers the bilayer to hexagonal (H_{11}) transition temperature for more unsaturated species. This is at variance with the observation that equimolar cholesterol induces the hexagonal (H_{11}) phase in the region 80–90°C for 12 : 0/12 : 0 phosphatidylethanolamine (data not shown), but such observations are complicated by the much shorter chain length of the dilauroyl species which may also encourage the adoption of non-bilayer phases. In general the results indicate that for pure phosphatidylethanolamine cholesterol does not induce dramatic effects on the polymorphic phase behaviour, is easily incorporated into the hexagonal (H_{11}) phase, and may

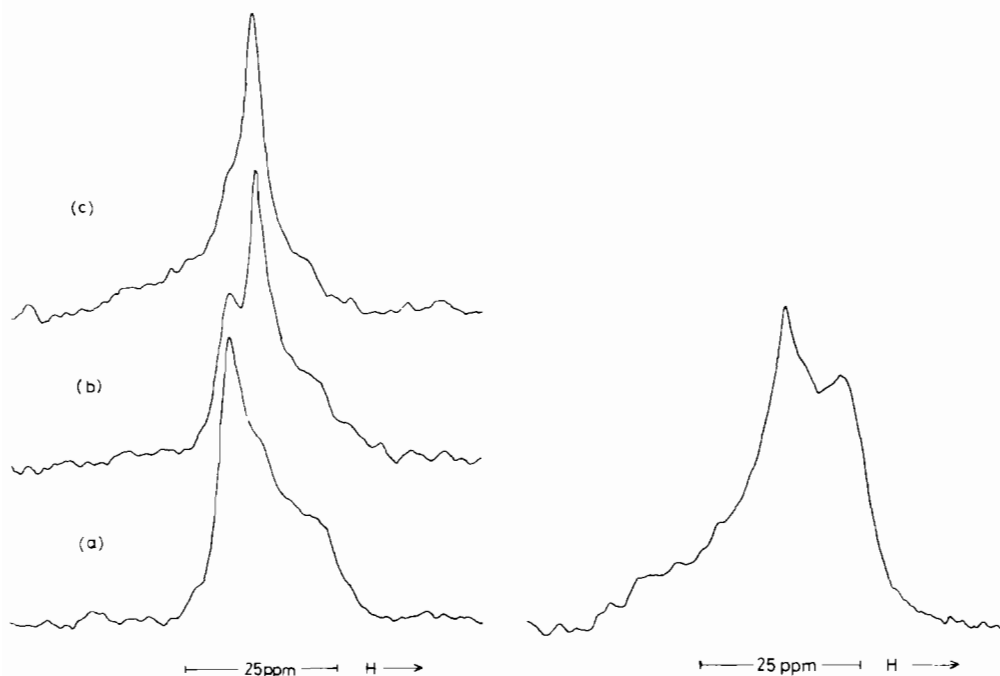


Fig. 6. 36.4 MHz ^{31}P NMR spectra of aqueous dispersions of egg yolk phosphatidylethanolamine at 40°C as a function of p^2H . a, p^2H 7.0; b, p^2H 9.0; c, p^2H 10.0.

Fig. 7. 36.4 MHz ^{31}P NMR spectra of egg yolk phosphatidylethanolamine at 40°C , dispersed in 50 mM borate/NaOH, 100 mM NaCl (p^2H 10.0).

lower the temperature somewhat (up to 10°C) at which the hexagonal (H_{11}) phase is first observed.

The influence of monovalent and divalent cation concentrations and the p^2H of the aqueous medium on the phase behaviour of phosphatidylethanolamines were also investigated, employing the species isolated from egg yolk. It was found that the introduction of up to 100 mM NaCl or 10 mM CaCl_2 had no appreciable effect on the temperature-dependent polymorphic phase behaviour. Changes in the p^2H did, however, have strong effects as detailed in Fig. 6. The ^{31}P NMR spectra depicted there were obtained at 40°C for a sample initially at p^2H 7.0, where the p^2H was raised incrementally by adding aliquots of 0.1 M NaOH while vortexing. At pH 9.0 a component of the lipid in a phase allowing isotropic motional averaging is observed (cubic, rhombic or inverted micellar, see ref. 5) as indicated by the narrow spectral component in Fig. 6b. At pH 10.0 (Fig. 6c) the hexagonal (H_{11}) phase is almost completely inhibited, and a substantial fraction of the lipid has adopted the bilayer phase, as indicated by the broad spectral component with a low field shoulder. The preference of egg yolk phosphatidylethanolamine for the bilayer phase at these higher pH values is particularly obvious when the lipid is initially hydrated at $\text{pH} > 9.0$. As illustrated in Fig. 7, the ^{31}P NMR spectra obtained for egg yolk phosphatidylethanolamine hydrated in a buffer at pH 10.0 indicates that fully 90% of the lipid enjoys the bilayer phase, with a small component in the phase allowing isotropic motional averaging.

Discussion

The major result of this investigation is that unsaturated phosphatidylethanolamines from both natural and synthetic sources undergo well defined, coherent bilayer to hexagonal (H_{11}) phase transitions at a characteristic temperature which is sensitive to the fatty acid composition. This transition occurs within 15°C of the high temperature end of the hydrocarbon phase transition. These observations considerably extend our understanding of the physical properties of phosphatidylethanolamines and have direct implications for the properties of phosphatidylethanolamines in biological membranes.

In this regard, one of the motivations for this study was that the predilection of unsaturated phosphatidylethanolamines for non-bilayer phases could not be reconciled with bilayer structure of such membranes as the outer membrane of *E. coli*, where 85% of the membrane lipids are phosphatidylethanolamines [15]. The results presented here suggest that *E. coli* compensates for the tendency of phosphatidylethanolamines to adopt the H_{11} phase by decreasing the degree of unsaturation of the constituent fatty acids, thus raising the hydrocarbon phase transition temperature and, concomitantly, the bilayer to hexagonal (H_{11}) transition temperature. In this context it is possible that the changes in the fatty acid compositions of membranes of *E. coli* grown at different temperatures [16] do not reflect a need to maintain a certain fluidity in the hydrocarbon region, but rather reflect the need to maintain the bilayer structure of the membrane, which is of course vital to cellular integrity.

The observed polymorphic phase behaviour of the two phosphatidylethanolamines isolated from mammalian sources also have important implications. The most obvious of these is that at physiological temperatures these components prefer non-bilayer (hexagonal H_{11} phase) structure, and will therefore tend to destabilize bilayer structure. The situation in the erythrocyte membrane, where the phospholipid species are asymmetrically distributed across the bilayer [17], is of particular interest. At physiological temperatures the inner monolayer, which is comprised of 49% phosphatidylethanolamine, 25% phosphatidylserine, 12% phosphatidylcholine and 12% sphingomyelin [16] must be considered to be somewhat unstable. The additional observation that Ca^{2+} can trigger isothermal bilayer to hexagonal (H_{11}) transitions in mixtures of phosphatidylethanolamine and phosphatidylserine (see reference 26) further suggests that this instability is likely to be expressed when the intracellular concentration of Ca^{2+} is raised, which may occur either as a result of ATP depletion or by introducing Ca^{2+} ionophores. Thus it may be immediately speculated that the 'blebbing off' of small vesicles from erythrocytes on ATP depletion [18] as well as simultaneous pronounced changes in erythrocyte morphology [19] may arise because of the tendency of the lipids in the inner monolayer to adopt non-bilayer configurations. In this regard the occurrence of intramembrane inverted phases as depicted in Fig. 6c of ref. 5 may serve as nucleation points for formation of the ejected vesicles, as well as serving to reduce the number of lipids actually comprising the inner monolayer, thus producing the observed morphological changes. A related point is that the appearance of phosphatidylethanolamine in the outer monolayer of ATP-depleted erythrocytes [20] may correspond to an enhanced 'flip-flop' rate, via the model suggested elsewhere

[5], arising from the facilitated formation of intramembrane inverted lipid configurations. These possibilities are currently under active investigation.

The results presented here show clearly that the presence of at least trans mono-unsaturated liquid crystalline acyl groups are required to enable phosphatidylethanolamines to adopt the hexagonal (H_{11}) phase. The requirement for the liquid crystalline state is fully consistent with early X-ray studies [21] where it is pointed out that gel state phospholipids are always found in the bilayer configuration, presumably due to packing constraints in the hydrocarbon region. Similarly, the requirement of a minimal degree of unsaturation is consistent with previous considerations [5] that the lipid molecule must exhibit a dynamic 'wedge' or 'cone' shape (where the polar region is at the smaller end of the cone) in order to assume such 'inverted' phases as the hexagonal (H_{11}). Finally, the observation that cholesterol is easily incorporated into the hexagonal H_{11} phase and may encourage its formation confirms previous studies [5,2] indicating cholesterol has a similar cone shape. However, the results presented here and elsewhere [5,7] show clearly that cholesterol exerts its most dramatic effects in mixed lipid systems containing unsaturated phosphatidylethanolamines and phosphatidylcholines, where it very actively promotes the formation of alternative phases such as the hexagonal (H_{11}) phase.

The influence of pH on the polymorphic phase behaviour of egg phosphatidylethanolamine clarifies some of the rather confused literature concerning the behaviour of phosphatidylethanolamines in model membrane systems. In particular, the observation that the bilayer phase is stabilized at pH values above 9 is consistent with an ability to form liposomal multibilayer structures at such pH values [23] as well as the small unilamellar sonicated vesicles [24]. In this regard the observation of an intermediary phase on raising the pH through 9.0, which is characterized by the possibility of isotropic motional averaging is very similar to behaviour observed elsewhere [5,7] for lipids progressing from an hexagonal H_{11} configuration to the bilayer phase or vice-versa as the temperature is varied. X-ray studies [21] also report the presence of such intermediary phases, which may have cubic, rhombic or tetragonal [21] structure, and also show that the area per lipid molecule at the hydrocarbon-water interface increases as the phospholipid assumes the hexagonal, 'intermediate', and bilayer phases in turn. Thus increasing the pH corresponds to increasing the area/molecule in the polar region, which would be entirely consistent with previous suggestions that higher pH values eliminate the possibility of transitory intermolecular hydrogen bonding between the polar headgroups of phosphatidylethanolamines [23].

In summary, phosphatidylethanolamines have remarkable properties, the most dramatic being their tendency to adopt the hexagonal (H_{11}) phase when in the liquid crystalline state. Such behaviour provides new insight into the dynamic structural and functional properties of lipids in biomembranes. In cases where phosphatidylethanolamines are a majority species it would appear that the fatty acid composition is adjusted to obtain maximal fluidity while ensuring the bilayer phase is maintained. Alternatively, in mammalian membranes phosphatidylethanolamines are a member of a class of lipids which act to destabilize bilayer structure and promote the formation of alternative

phases. The occurrence and modulation of such phases could lie at the heart of many biomembrane properties, ranging from fusion [6] to 'flip-flop' [5] to the action of anaesthetics [26].

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