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Rapid Report

N-Acylethanolamines as membrane topological stress compromising agents

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The effect of different *N*-acylethanolamines on the phase behaviour of fully hydrated egg phosphatidylethanolamines is reported. In particular, in the presence of *N*-acylethanolamines, the transition from the liquid-crystalline lamellar ($L\alpha$) to the inverse hexagonal (H_{II}) phase is observed at higher temperature with respect to the temperature transition of pure phosphatidylethanolamine. Moreover, in correspondence of this transition, an intermediate Q^{224} (space group Pn3m) cubic phase has been detected. Since the structure of this cubic phase presents unique topological analogies with the lipid bilayer organization, these data suggest the possible role of *N*-acylethanolamines in stabilizing the biological membranes by avoiding a sudden change to a non-bilayer phase in those tissues which undergo stress conditions.

Long chain *N*-acylethanolamines (NAEAs) have been found at relatively high levels of 400–500 nmol per g tissue in the infarcted area of canine myocardium [1]. An unusual molecule (phospholipid X) was isolated as a major component of total phospholipids of granular cells from pig and human epidermis [2] and subsequently identified as phosphatidyl-(*N*-acyl)-ethanolamine, a precursor of long-chain NAEAs [3]. Since phosphatidyl-(*N*-acyl)-ethanolamines are present in granular cells but not in the basal and spinous cells [3], the possibility exists that nature created some unknown biological role for catabolic products of these phospholipids including NAEAs.

The passage of epidermal cells from the basal layer to the outer surface of the stratum corneum is associated with an increase in various catabolic activities. All phospholipids are catabolized, since the cells at the stratum corneum do not contain phospholipids [3]. By comparing heart and granular cells, we can imagine some common stress factors following the synthesis of NAEAs, since catabolic processes predominate both in the heart necrotic region and in the surface layer of the skin. It has been reported that NAEAs, specifically *N*-oleoylethanolamine, can stabilize mitochondrial membranes against a nonspecific permeability increase caused by Ca^{2+}-releasing agents [4]. The mechanism by which NAEAs perform such an action is a matter of considerable interest. It was suggested that *N*-acylethanolamines interfere with Ca^{2+} binding [4], but other explanations also appear possible. In particular, the chemical characteristics of the NAEA molecules suggest that a primary action may be played on the lipidic part of the mitochondrial membrane.

Lipids isolated from biological membranes have long been known to form lamellar phases when mixed with water; however, naturally occurring lipids can form even non-lamellar phases [5–9]. In particular, 'stress' conditions, including an increase in temperature, less saturation of phospholipid hydrocarbon chains, dehydration of the system or increase in concentration of some divalent cations, especially Ca^{2+}, may induce the appearance of hexagonal and cubic phases [5–10]. Fully hydrated egg phosphatydilethanolamine (egg-PE) exhibits three different phases, i.e., the gel, the liquid-crystalline lamellar $L\alpha$ and the inverted hexagonal H_{II} phases, as a function of the temperature [8,11–13]. In this study, we investigated the effect of NAEAs on the phase behaviour of fully-hydrated egg-PE by differential scanning calorimetry (DSC), X-ray diffraction and

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steady-state anisotropy of trimethylammonium diphenylhexatriene (TMA-DPH) fluorescence (fluorescence spectroscopy).

N-Acylethanolamines were synthesized as previously described [14]. Fluorescence measurements were performed according to Ambrosini et al. [15]. Samples for DSC and X-ray diffraction experiments were obtained as follows: aliquots of an egg-PE chloroform solution were mixed in a test tube with the desired amounts of chloroform NAEA solutions. The solvent was removed from the NAEA/egg-PE mixture by nitrogen drying and then the samples were kept under vacuum for several hours. The lipid films were rehydrated in a buffer of 10 mM sodium citrate, 150 mM NaCl, 0.1 mM EDTA (pH 5) above the hydrocarbon chain-melting transition of egg-PE [16]. For all samples, a lipid-to-buffer weight ratio of 1:3 was used. The investigated NEAE/egg-PE molar ratio was 0.2. Differential scanning calorimetry measurements were performed by using a Perkin-Elmer DSC2 calorimeter equipped with a thermal analysis data station. Heating scans were performed at 5C°/min between 0°C and 80°C. Aluminium containers of 20 μl capacity were used. For X-ray diffraction experiments, a conventional X-ray generator, equipped with a Guinier camera operating in vacuum with a bent quartz crystal monochromator (λ = 1.54 Å) and a cylindrical film cassette with diameter of 123 mm, has been used. The samples were mounted in vacuum tight cells with thin mica windows.

DSC (Fig. 1) and fluorescence measurements (data not shown) indicate that in pure fully hydrated egg-PE, the gel to Lα phase transition occurs approx. at 15°C, while the Lα to H_{II} phase transition appears broad and centered at about 50°C. The analysis of the X-ray diffraction patterns recorded at different temperatures

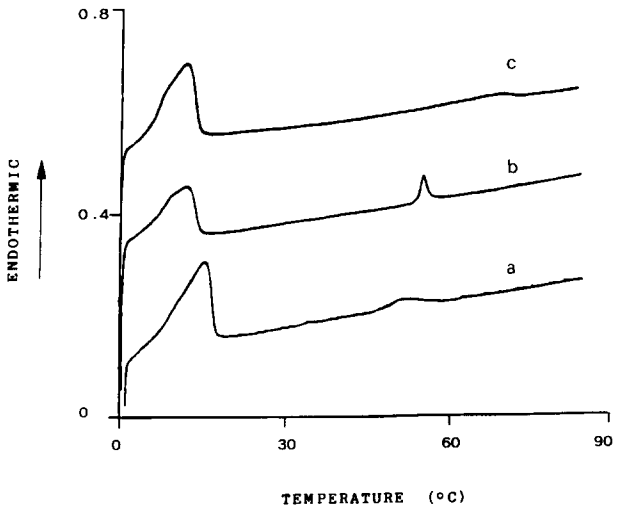

Fig. 1. DSC heating scans of fully hydrated control egg-PE (a), egg-PE containing *N*-oleoylethanolamine (NOEA) 0.2 molar ratio (b) and egg-PE containing *N*-lauroylethanolamine (NLEA) 0.2 molar ratio (c).

TABLE I

X-ray diffraction: experimental data

In each X-ray diffraction experiment, a number of sharp low-angle reflections are observed, and their spacing measured. The different phases are identified by analysing the spacing ratios of the observed reflections [5,7,17]. The unit cell dimensions are then obtained by using the Bragg law. In the case of the cubic phase, the observed reflections specify the extinction symbol [5,17]: the structure belongs to the cubic aspect 4. Only two space groups are compatible with the observed reflections: according to previous papers [5,6], the one with highest symmetry (No. 224) has been adopted.

Temp. (°C)	Phase	Unit cell parameter (Å)
Control egg-PE		
30	Lα	48.3
50	Lα/H_{II}	47.8/70.8
60	H_{II}	71.5
70	H_{II}	69.5
80	H_{II}	67.8
Egg-PE/NLEA		
30	Lα	49.0
50	Lα	48.2
60	Lα	47.6
70	H_{II}/Q^{224}	67.9/130.6
80	H_{II}/Q^{224}	66.3/124.7
Egg-PE/NOEA		
30	Lα	49.4
50	Lα	47.9
60	H_{II}/Q^{224}	70.7/137.7
70	H_{II}/Q^{224}	67.8/129.1
80	H_{II}	66.6

confirms this observation: some structural data relative to these phases are reported in Table I. When *N*-lauroylethanolamine (NLEA) and *N*-oleoylethanolamine (NOEA) are added to fully hydrated egg-PE systems, the DSC curves appear slightly modified; however, new peaks do not occur. In agreement with previous results obtained by fluorescence on dipalmitoylphosphatidylcholine liposomes (Ambrosini et al., unpublished data), DSC (Fig. 1) and fluorescence data (not shown) indicate that both NAEAs decrease the gel to Lα phase-transition temperature. Moreover, in DSC curves, the addition of NLEA to egg-PE in a molar ratio of 0.2, determines a 13°C shift in the position of the endothermic broad peak, the Lα to H_{II} phase transition being now observed at about 68°C. When NOEA is added to fully hydrated egg-PE with the same molar ratio, the endothermic peak appears narrow, but again shifted to higher temperatures (about 5°C). The fluorescence data did not reveal such phenomena.

X-ray diffraction experiments show that the addition of 0.2 molar ratio NAEA to fully hydrated egg-PE determines the occurrence of a more complicated polymorphism. At 30°C the lamellar Lα phase is still observed with structural parameters quite similar to those observed in the pure egg-PE system (Table I).

However, in correspondence of the endothermic peak observed on DSC trace, the X-ray diffraction data indicate that NOEA induces the occurrence of a very large region, where almost two different phases coexist in equilibrium. In this region, the X-ray diffraction profiles are characterized by a large number of narrow Bragg peaks, the indexing of which is consistent with the presence of a structure of cubic Pn3m symmetry. The corresponding spacing ratios are reported in Table II. In particular, at temperature just below the calorimetric peak maximum, the cubic structure coexists with the lamellar phase, while at higher temperatures, a hexagonal phase replaces the lamellar one. When the temperature is further increased, the peaks related to the hexagonal structure appear more and more intense, while those related to the cubic phase become

TABLE II

X-ray diffraction: examples of space group analysis

s_{obs}, s_{lam}, s_{hex} and s_{cub} are the reciprocal spacings in $Å^{-1}$ ($s = 2 \sin \theta / \lambda$, where 2θ is the scattering angle) of the observed reflections and the ones respectively calculated for a 1-D lamellar, 2-D hexagonal and 3-D primitive cubic lattices [7,17]. The following equations have been used: $s_{lam} = h/a$, $s_{hex} = (2/a\sqrt{3})(h^2 + k^2 + hk)^{-1/2}$ and $s_{cub} = (1/a)(h^2 + k^2 + l^2)^{-1/2}$, where h, k and l are the Miller index of the reflections and a is the unit cell parameter reported in Table I.

Control egg-PE:

30°C			50°C						60°C			
Lα, a = 48.3 Å			Lα, a = 47.8 Å; H_{II}, a = 70.8 Å						H_{II}, a = 71.5 Å			
s_{obs}	h	s_{lam}	s_{obs}	h	s_{lam}	h	k	s_{hex}	s_{obs}	h	k	s_{hex}
0.0206	1	0.0207	0.0164			1	0	0.0163	0.0161	1	0	0.0161
0.0411	2	0.0414	0.0211	1	0.0209				0.0278	1	1	0.0280
0.0618	3	0.0621	0.0285			1	1	0.0282	0.0324	2	0	0.0323
0.0832	4	0.0827	0.0322			2	0	0.0326	0.0427	2	1	0.0427
			–	2	0.0418				0.0482	3	0	0.0484
			0.0621	3	0.0628							

Egg-PE/NLEA:

60°C			70°C							
Lα, a = 47.6 Å			H_{II}, a = 67.9Å; Q^{224}, a = 130.6 Å							
s_{obs}	h	s_{lam}	s_{obs}	h	k	s_{hex}	h	k	l	s_{cub}
0.0208	1	0.0210	0.0108				1	1	0	0.0108
0.0419	2	0.0420	0.0135				1	1	1	0.0133
0.0631	3	0.0630	0.0154				2	0	0	0.0153
0.0839	4	0.0840	0.0169	1	0	0.0170				
			–				2	1	1	0.0188
			0.0216				2	2	0	0.0217
			0.0227				2	2	1	0.0230
			0.0295	1	1	0.0295				
			0.0340	2	0	0.0340				
			0.0453	2	1	0.0450				

Egg-PE/NOEA:

50°C			60°C								80°C			
Lα, a = 47.9 Å			H_{II}, a = 70.7 Å; Q^{224}, a = 137.7 Å								H_{II}, a = 66.6 Å			
s_{obs}	h	s_{lam}	s_{obs}	h	k	s_{hex}	h	k	l	s_{cub}	s_{obs}	h	k	s_{hex}
0.0208	1	0.0209	0.0103				1	1	0	0.0103	0.0174	1	0	0.0173
0.0417	2	0.0418	0.0127				1	1	1	0.0126	0.0298	1	1	0.0300
0.0630	3	0.0626	0.0145				2	0	0	0.0145	0.0348	2	0	0.0347
0.0836	4	0.0835	0.0164	1	0	0.0163								
			0.0174				2	1	1	0.0178				
			0.0203				2	2	0	0.0205				
			0.0218				2	2	1	0.0218				
			–				3	1	0	0.0230				
			–				3	1	1	0.0241				
			0.0251				2	2	2	0.0252				
			0.0282	1	1	0.0283								
			0.0327	2	0	0.0327								

less visible. At about 80°C, only the hexagonal phase is observed; this behaviour suggests a continuous and gradual transiton from the cubic to the hexagonal structure. A quite similar structural behaviour is also observed in the NLEA system, the only difference being in the temperature range where the biphasic region extends. In particular, at 80°C, both the cubic and hexagonal phases are still present. It must be stressed that severe difficulties were encountered in trying to resolve the biphasic region of the phase diagram, probably due to metastable effects with the cubic phase. Moreover, the presence of the cubic phase in correspondence and largely above the phase transition temperature could explain the lack of significant modifications in the fluorescence anisotropy values.

The structure of the Pn3m cubic phase (Q^{224} in our notation [5]) has been described using different notions, referring to its rod skeleton [5] or considering surfaces of minimal mean curvature [18,19] or, very recently, in terms of an orderly space distribution of short-range conformational disorder [6]. For the sake of simplicity, the Q^{224} structure is described here as consisting of a pair of disjointed three-dimensional labyrinths, mutually intertwined and unconnected. Each labyrinth is a three-dimensional network of identical rods; the rod skeleton structure is represented in Fig. 2. So far, the Q^{224} cubic phase has been reported in a variety of lipid systems [5,6,8,20], but examples are known of only type II: the two labyrinths are indeed filled by water and are coated by the lipid polar groups, whilst the interstices beetween the rods are occupied by the hydrocarbon medium. Considering that in PE systems the hexagonal phase is type II [8], the inverse structure appears the most probable one also for the induced cubic phase. Moreover, the structural parameters, which could be determined from the lattice dimensions and the chemical composition (estimated by gravimetric analysis, presuming that in the biphasic region the water concentration of the sample coincides with the water phase concentration) seem to confirm this picture. The dimensions of the structure elements, calculated by using the equations reported in Refs. 5 and 21, are reported in Table III. In the limit of the assumed composition, they appear compatible only with a type II structure.

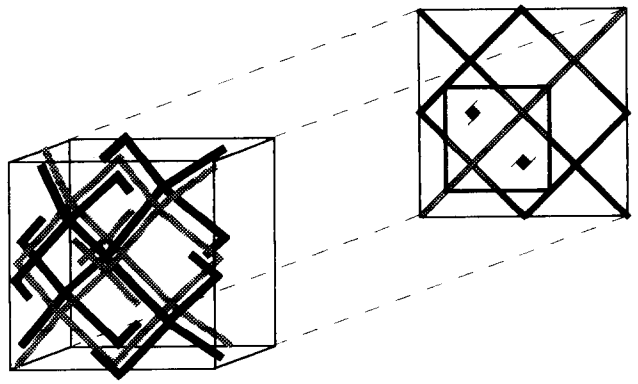

Fig. 2. Representation of the structure of the Q^{224} cubic phase: Pn3m, centrosymmetric, origin at centre (3m) [17]. The structure, represented by a skeleton of rods, consists of two three-dimensional networks of rods, mutually intertwined and unconnected. The rods are joined tetrahedrally four by four. The rods occupy position e, of symmetry (3m), the junctions position a, of symmetry (43m), being the special positions labelled as in International Tables for Crystallography [17]. The black heavy lines mark the projection of the unit cell; some of the symmetry elements are shown. The content of the box (defined by the light lines) is shown in projection and in perspective.

Q^{224} belongs to the bicontinuous cubic phases [5,6,9]: in its structure, the three mutually disjointed media, two polar and one apolar, are continuous throughout the three-dimensional space. This phase may be considered as a topological generalization of the paradigm of the lipid organization, namely the bilayer, where a

TABLE III

Q^{224} cubic phase: dimensions of the structure elements

c_{wat} is the water weight concentration of the phase. a is the lattice parameter (see Table I). $C_{v,pol}$ is the volume concentration of the polar moiety (water and polar headgroups of the lipid molecules), calculated considering the mean chemical composition of the egg-PE [22] and the values of 1.00 and 0.99 cm^3/g as specific volumes at 20°C for the water and for both egg-PE [11] and NAEA compounds, respectively. Volumes have been all reduced at the reported temperatures, by using the thermal coefficient found by Seddon et al. [23]. The structure elements are supposed to be straight rods of circular section, length L_{rod} and radius R_{rod}. S_{ch} is the area-per-chain at the polar/apolar interface [5]. The indices I and II refer to the hypothesis of type I and type II structures. $R_{rod,I}$ ($R_{rod,II}$) is then the radius of the hydrocarbon (polar) core of the rods. The length of fully-extended hydrocarbon chains in the egg-PE system, as measured from molecular models, ranges from about 19 to 23 Å: in type-I structures, this length is bound to be larger than R_{rod}.

T (°C)	a (Å)	$C_{v,pol}$	L_{rod} (Å)	$R_{rod,II}$ (Å)	$S_{ch,II}$ (Å^2)	$R_{rod,I}$ (Å)	$S_{ch,I}$ (Å^2)
Egg-PE/NLEA (c_{wat} = 50% (w/w))							
70	130.6	0.612	113.1	35.7	36.8	27.4	31.6
80	124.7	0.586	107.9	33.2	48.4	27.1	34.1
Egg-PE/NOEA (c_{wat} = 55% (w/w))							
60	137.7	0.651	119.3	39.0	39.2	27.2	31.7
70	129.1	0.650	111.8	36.6	42.1	25.5	34.1

unique and continuous hydrocarbon septum separates two continuous and disjoined water media. In previous papers, it was suggested that a possible biological role of bicontinuous cubic phases was the preservation in the cell of the separation between the inter- and the intra-cellular media, essential for survival [5,6]. Infinite polar lamellae (i.e., the biological membrane) may be destabilized by 'stress' factors whenever the steric requirements of the polar headgroups and of the hydrocarbon chains cannot both be satisfied at once. One way to release the constraints is to fragment the lamellae into straight rods, ribbons or disks. However, if the functional distinction between a cell's 'inside' and 'outside' must be preserved, the structural frustrations could be indeed reduced only by folding the lipid lamella in a cubic bicontinuous lattice. The observation of such a structure in lipid systems containing *N*-acylethanolamines suggests that these lipid derivatives may have a protective effect against damages induced by stress conditions (e.g., cellular necrosis), by avoiding the sudden change from bilayer to non-lamellar phases.

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