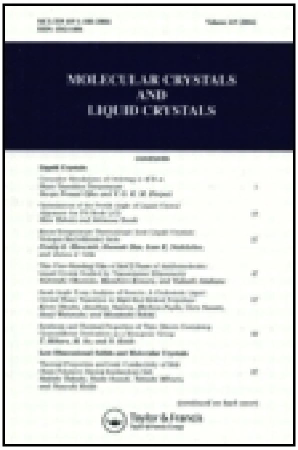

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Measurement of Forces in Lamellar and Hexagonal Phases of Alkyl Esters of Acylcarnitine by Osmotic Stress Technique

Paolo Mariani [a] & Letizia Saturni [a]
[a] Istituto di Scienze Fisiche, Facoltà di Medicina e Chirurgia, Università and Istituto Nazionale per la Fisica della Materia, INFM, research unit of Ancona, via Ranieri 65, I-60131, Ancona, Italy

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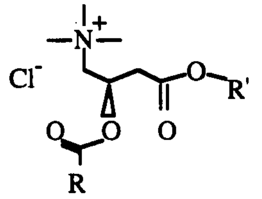
MEASUREMENT OF FORCES IN LAMELLAR AND HEXAGONAL PHASES OF ALKYL ESTERS OF ACYLCARNITINE BY OSMOTIC STRESS TECHNIQUE

PAOLO MARIANI and LETIZIA SATURNI
Istituto di Scienze Fisiche, Facoltà di Medicina e Chirurgia,
Università and Istituto Nazionale per la Fisica della Materia,
INFM, research unit of Ancona, via Ranieri 65, I-60131
Ancona (Italy).

Abstract It has been recently reported that alkyl esters of acyl-L-carnitine show a lyotropic liquid crystalline polymorphism. Depending on the relative length of the hydrocarbon chains, two different phase behaviours were detected: in addition to the lamellar Lα phase, compounds bearing a relatively short alkyl chain exhibit hexagonal H and cubic Ia3d type I phases. In this paper, X-ray diffraction and osmotic stress experiments have been performed in order to directly measure as a function of distance the forces between structure elements, i.e., lamellae in the Lα phase and rods in the H phase. In both cases, exponentially decaying repulsive forces dominate the interactions at close separation. The magnitude and decay length of these forces are very similar. The exponential nature of the repulsion and the value of the decay length led us to conclude that the dominant force experienced by the structure element surfaces approaching contact is hydration repulsion. This force represents the work of removing water from the vicinity of the approaching surfaces.

INTRODUCTION

Acyl-L-carnitines (acyl esters of the (R)-3-hydroxy-4-trimethyl ammonium butanoate) are involved in the transport of fatty acid across the inner mitochondrial membrane[1,2]. Such molecules are amphiphilic, are very surface active and form micellar phases[3]. Similar properties has been also observed in long chain acylcarnitine esters,



which have been also proved to possess anti microbial activity[4].

Recently, it has been demonstrated that acylcarnitine alkyl esters exhibit a lyotropic polymorphism[5]. As a function of concentration, two different phase sequences were observed (see figure 1). Intermediate between the isotropic solution and the lamellar Lα phase, where lipid molecules assemble into stacked layers separated by water, some derivatives form hexagonal H and cubic Ia3d (Q^{230}, in our notation[6]) phases. Such phases are non-lamellar: the H phase consists of cylindrical structure elements packed on a 2-D hexagonal lattice, while the cubic Q^{230} is bicontinuous and consists of a pair of 3-D disjoined labyrinths, related to each other by an inversion centre[6]. In the present case, both the non-lamellar phases are type I (direct)[5]: the structure elements are filled by

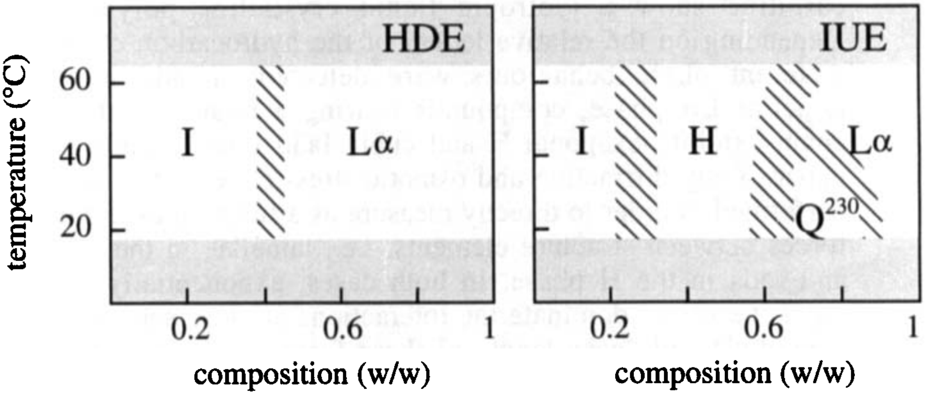


FIGURE 1 Composition-temperature phase diagrams of the two representative compounds HDE and IUE as deduced from previous X-ray diffraction measurements on gravimetrically prepared samples[5]. I isotropic, Lα lamellar, H hexagonal, and Q^{230} cubic phases. Hatched regions were not investigated.

the hydrocarbon chains and are embedded in the water matrix, the carnitine polar groups lying on the water/paraffin interface. The ability of the different investigated acylcarnitine esters to form non-lamellar phases was related to the length of the two hydrocarbon chains[5]. Curved, convex interfaces (as in the type I non-lamellar phases) are possible only for derivatives which have a relatively short alkyl chain and behave like single chain surfactants; by contrast, compounds which have a relatively long alkyl chain are effective double chain surfactants and then generate only quasi-planar interfaces (as in the lamellar phase).

Indeed, a knowledge of the interaction force between the structure elements and of its dependence on the distance between the structure elements, is necessary for understanding how the stability required for the formation of the different phases is controlled. In fact, distance-dependent interactions, and in particular hydration forces, have been recognised to create strong and specific associations, and to act as a key to the regulation of the structural properties of macromolecular assemblies[7]. In this paper, X-ray diffraction and osmotic stress[8] have been used to directly measure the forces between the structure elements in the lyotropic phases of acylcarnitine esters. Considering the phase behaviour[5] (figure 1), two representative derivatives have been analysed, namely the isovaleryl-L-carnitine undecyl ester chloride ($R=isoC_4H_9$, $R'=C_{11}H_{23}$, IUE) and the heptanoyl-L-carnitine dodecyl ester chloride ($R=C_6H_{13}$, $R'=C_{12}H_{25}$, HDE).

In particular, osmotic stress measurements have been performed on the basis of the observation that acylcarnitine esters in water solution, when exposed to a polymer such as polyethylene glycol (PEG), condenses into a lyotropic phase separate from the polymer solution. The condensed phase can be analysed by X-ray diffraction and the separation between the structure elements measured. Under conditions in which the polymer is excluded from the lattice, the osmotic pressure of the polymer solution is the osmotic stress compressing the lattice. The force of compression is equal and opposite to the repulsion between the structure elements at the observed separation. As the osmotic pressure of the solution can be determined from the polymer concentration, forces between lipid bilayers in the lamellar $L\alpha$ phase and between rods in the H phase have been derived.

MATERIALS AND METHOD

The method for direct force measurements by osmotic stress has been described in details by Parsegian and co-workers in several papers[7-11], to which the reader should make reference for more information.

Acylcarnitine esters were obtained as previously reported[5]. Polyethylene glycol, PEG, 15000-20000 MW was of commercial origin (Sigma). Samples were prepared by equilibrating the acylcarnitine ester phase against PEG water solutions in vast excess. Condensed phases were obtained in PEG solutions of at least 5 wt%. Equilibrium conditions, in which the lattice spacing of the condensed phase resulted independent of time, were usually reached in 24 h.

Osmotic pressures π of the solutions were calculated from PEG weight concentration using equations reported by Rand and co-workers[8].

X-ray diffraction experiments were performed using a Philips PW 1830 X-ray generator equipped with a Guinier-type focusing camera operating in vacuum. The CuKα_1 line isolated by a bent quartz crystal monochromator was used. Diffraction patterns were recorded on Kodak DEF-392 films. Samples were held in a vacuum tight cylindrical cell provided with thin mica windows.

RESULTS

Acylcarnitine ester samples in which the water stress was measured, were analysed by X-ray diffraction to determine the structure and the unit cell dimension of the condensed phase. Following the usual procedure[6,12], the structure was identified analysing the spacing ratios of the low-angle diffraction peaks, which are expected to be in the order 1:2:3:4... and $1:\sqrt{3}:\sqrt{4}:\sqrt{7}$... for the L$\alpha$ and the H phase, respectively[12]. In each experiment, from 3 to 5 Bragg peaks were observed, which assure a correct assignment of the structure: the low experimental resolution (about 10-15Å) should not surprise, as it is due to the short-range disorder which is a feature of the lyotropic phases[6,12].

For the HDE compound, PEG solutions up to 40 wt% determined the condensation of a lamellar Lα phase. By contrast, in the case of IUE derivative, a hexagonal phase condensed from PEG solutions up to 38 wt%, while a cubic Q^{230} phase appeared at higher polymer concentrations. From the peak spacings, the unit cell dimension of the condensed phases was calculated: it should be noticed that in the lamellar and hexagonal phases, this dimension corresponds to the distance between the mid planes of lipid bilayers (d), and to the interaxial distance between the rods (a), respectively (see the inserts in figure 2). Figure 2 shows that both distances decrease monotonically with increasing the osmotic pressure of the polymer solution. By referring to equivalent unit cell dimensions of the gravimetrically prepared samples reported by De Maria and co-workers[5], the composition of the condensed phases were then derived. The upper scale of the graphs in the figure 2 indicates this estimated composition. Assuming as usual that water is completely excluded from the paraffinic regions[6,12] and using the equations reported by Luzzati[5,12], the bilayer separation d_w and the lipid layer thickness d_l in the $L\alpha$ phase, and the radius of the lipid rods R_l and the intercylinder water thickness d_{wi} in the H phase were also calculated (such parameters are defined in the inserts of figure 2). Their variation with PEG concentration is reported in figure 2.

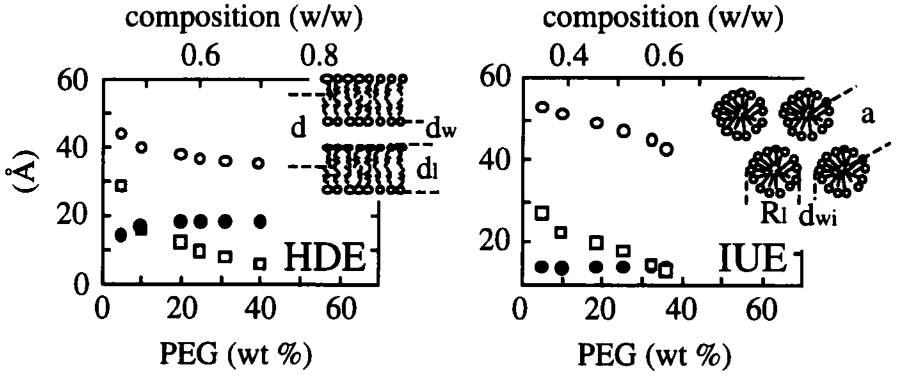


FIGURE 2 Variation of the interlamellar and interaxial distances (d and a, open circles), bilayer and intercylinder separations (d_w, and d_{wi}, open square), and lipid layer thickness and lipid rod diameter (d_l and R_l, closed circles) with PEG concentration for HDE and IUE compounds, respectively. The inserts show a schematic representation of the structures. The upper scale indicates the composition of the condensed phases, as it has been estimated comparing the unit cell dimension of the gravimetrically prepared samples previously analysed[5].

From the estimated composition, and according to Luzzati's formalism[12], the volume of water associated with each carnitine polar group, V_w, was then calculated. In figure 3 (left frame), its variation with osmotic pressure is reported. At the same osmotic pressure, the number of water molecules *per* polar group is larger for the hexagonal than for the lamellar phase. The difference is bigger at low pressure and reduces as far as the water activity decreases. However, the data align very well on a common curve when the volume of water *per* carnitine polar group is reported as a function of the estimated composition of the condensed phase (figure 3, right frame). In this case, the hydration behaviour of the two compounds appears independent of the structure, suggesting that this property only depends on the nature of the surface.

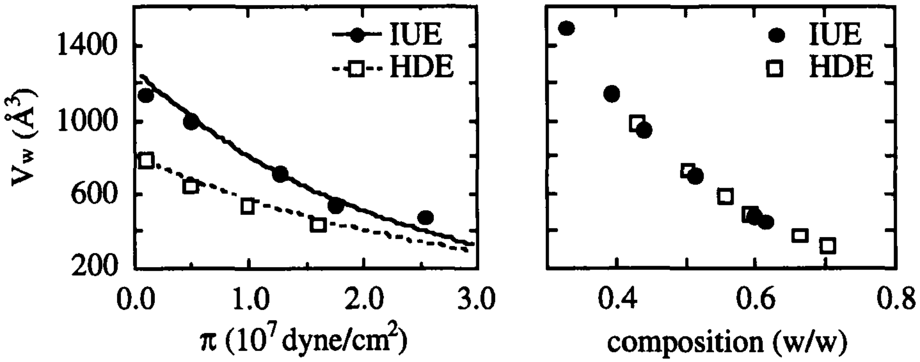


FIGURE 3 Variation of the volume of water *per* carnitine polar group as a function of the osmotic pressure π compressing the lattice (left frame) and as a function of the estimated composition of the condensed phase (right frame).

We then measured the energetic of dehydrating the lamellar and the hexagonal phases and forcing the structure elements closer together. Mechanically, in fact, the polymer solution exerts a pressure on the condensed phase, preventing the full swelling of the lattice: at equilibrium, and for each PEG concentration, repulsion between the structure elements is equal to the osmotic pressure of the solution. Figure 4 shows the force vs. distance curves obtained for the two investigated compounds. In the left frame, the osmotic pressure is reported as a function of the separation between the bilayers in the lamellar phase of HDE. It should be observed that for planar bilayers the osmotic pressure π and the repulsive force *per* unit area are equivalent[7]:

$$P(d_w) = \pi \qquad (1).$$

In the right frame of figure 4, the pressure is plotted against the interaxial distance between the rods in the hexagonal phase of IUE. The force *per* unit length between the nearest pair of rods, the interaction of which is taken to be pairwise additive, can be obtained by[10]:

$$f(a) = \pi\, a / \sqrt{3} \qquad (2).$$

Therefore, from the osmotic pressure π, the force between the structure elements could be calculated, and it is plotted as a function of the interaxial separation in the insert of figure 4.

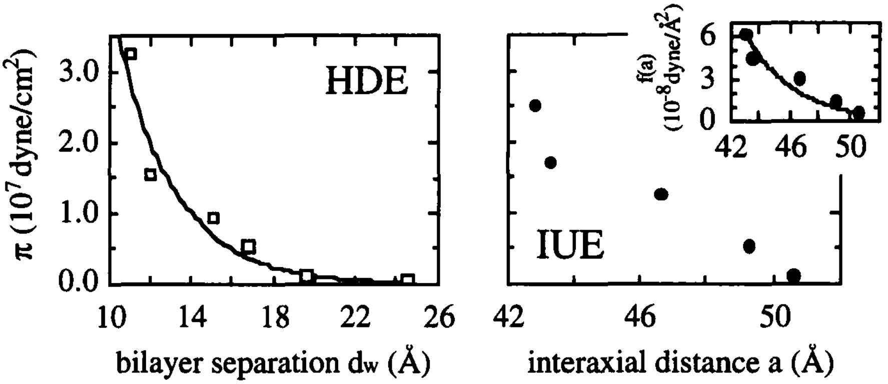


FIGURE 4 Dependence of the osmotic pressure π on the bilayer separation in the lamellar phase of HDE and on the interaxial distance in the hexagonal phase of IUE. The insert shows the force *per* unit length in the hexagonal phase calculated as reported in the text. Best fit curves relative to eq.s (3) and (4) are also reported: correlation coefficients as higher as 0.99 and 0.95 were obtained for HDE and IUE, respectively.

To choose the mathematical form for describing the force vs. separation curves, one must be aware of at least four different kinds of interactions expected to occur between the structure elements[7,8,13]: the hydration force due to perturbation of water by the polar surface; van der Waals attraction that limits hydration; repulsion due to thermal undulations of the whole structure elements; steric interactions of polar groups whose conformations are confined by an approaching surface. However, direct measurements of those interactions have shown that in the crucial last 15-20 Å of separation, hydration forces are far more important than the electrostatic, steric and van der Waals forces traditionally assumed to be dominant between charged surfaces[8-11,14,15]. For planar bilayers, such hydration repulsion decays exponentially with distance and has the following form[7]:

$$P\,(d_w) = P_0 \exp(-\, d_w / \lambda) \qquad (3).$$

For the hexagonal structure, the equivalent form is[11,14]:

$$f(a) = f_0 \exp(-\, a / \lambda) \qquad (4).$$

Hydration coefficients P_0 and f_0 reflect the degree to which the surface orders the boundary water. The decay distance λ is a property of the water itself, and reflects the way that ordering is propagated through water[13].

Theoretical correlations with the present data have been then obtained taking into account only hydration contributions. The force vs. distance curves of figure 4 have been analysed by fitting data with equations (3) and (4): the good correlation coefficients obtained in the fitting procedure strongly confirm such an analysis. In the case of the Lα phase of HDE compound, the repulsive force between the lamellae appears to fall off exponentially with a decay distance of 2.9 ± 0.3 Å. The force magnitude when the bilayer surfaces are at close contact P_0 is 1.3×10^9 dyne cm^{-2}. For the H phase of IUE, the repulsive force between the rods grows exponentially with 3.0 ± 0.4 Å characteristic distance as the cylinders are brought together. The f_0 parameter, calculated at close surface separation (i.e., for an interaxial distance of about 28Å) and rescaled in terms of intercylinder force per unit surface area, has a value of 5.1×10^9 dyne cm^{-2}.

DISCUSSION AND CONCLUSION

Although we do not exclude other interpretations, from these results it emerges that at small separations the structure elements in lyotropic phases of acylcarnitine esters show repulsive forces fully consistent with hydrational force expectations. Exponentially varying repulsive forces have been in fact demonstrated, both between cylinders in the hexagonal phase of IUE and between bilayers in the lamellar Lα phase of HDE. Decay lengths appear independent on the structure of the condensed phase and in very good agreement with the characteristic decay distances of 2-4 Å measured in neutral and charged lipids at surface distances lower than about 20 Å by Rand and co-workers[7-9,13,15]. Marcelja's theories based on the polarisation of the first water shells induced by surfaces or surface charged groups, predict an exponential hydration repulsion of unspecified decay length, which should be a property of water itself and not of the perturbing surfaces[13]. A value of the decay length around 2-3 Å, which corresponds to the size of a water molecule, has been considered to be reasonable. Because of the strong resemblance with the present situation, we then believe that the detected 3 Å decay-length exponential is a hydration force reflecting the work of removing water polarised on facing molecular surfaces.

It is also indicative that in both the investigated systems, not only the decay of the repulsive forces, i.e., the decay of perturbation of water away from the polar surface, but also the magnitude of the measured forces, i.e., the magnitude of perturbation of water at the polar surface, is very similar. Such observation allows the identification of the common source of interaction in the carnitine polar groups on apposing surfaces, the structural feature shared by the two investigated compounds. Therefore, while the phase behaviour has been clearly demonstrated to depend on the relative structural characteristic of the hydrocarbon chains[5], in the two investigated phases the interactions between the structure elements appear very similar, as they are dominated by the perturbation of water structure near the identical surface polar groups.

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