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# Study of Perturbations Induced by a Barbituric, the Phenobarbital, on DSPC Multilamellar Liposomes

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Multilamellar liposomes of distearoyl phosphatidyl choline in excess water condition, doped by the barbituric drug Phenobarbital, in molar ratios between 0 and 0.5, were studied by using differential scanning calorimetry, X-ray diffraction and electron microscopy. The results suggest that the drug interacts with the lipid headgroup region: in particular, at the higher concentrations investigated, an interdigitated "gel phase," with rigid chains, stiff and perpendicular to the lamellar planes, seems to be induced.

## INTRODUCTION

Barbiturates have a depressant effect on the activity of each excitable tissue; the central nervous system in particular shows an high sensitivity.[1] They may induce a central nervous system depression at all levels, from mild sedation to anesthesia.[2]

Barbiturates with a phenyl ring in the 5-position, such as phenobarbital (Figure 1), show a selective anticonvulsive effect.[3] This action leads to its wide use, in consideration that the prevalence of epilepsy is 5–6 per 1000 in West countries and phenobarbital represents a leading drug for chronic treatment schedule.

During chronic treatment phenobarbital reaches the steady state with slow fluctuations in the plasma levels. At present the mechanism by which phenobarbital

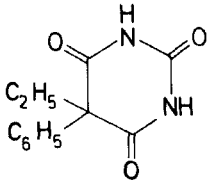



FIGURE 1 Chemical formula of the phenobarbital.



acts is not fully known. It has been shown that it interferes with biochemical pathways related to glucose metabolism, by inhibiting the energetic machinery. Moreover the selective anticonvulsive action of phenobarbital may result from its property of inhibiting the glutamate exciting effect and of increasing the gamma aminobutyric acid inhibiting effect.[4] The interaction with the cellular membrane is thought to play a fundamental role: in fact phenobarbital involves selective synaptic transmission and the long duration of its action may lead to a temporary structural modification. The described behaviour induced by phenobarbital may be related to a membrane-stabilizing effect: therefore we have studied the interaction of this compound with a model membrane made of distearoyl phosphatidylcholine, using different experimental techniques as differential scanning calorimetry (DSC), X-ray diffraction and transmission electron microscopy (TEM).

## MATERIALS AND METHODS

1,2-Distearoyl-3-sn-phosphatidylcholine (DSPC) and the phenobarbital drug were purchased respectively from SIGMA (St. Louis, Mo, USA) and from Bracco (Milano, Italy) and used without further purification.

### Sample Preparation

The lipid and the drug, in the fixed ratios, were dissolved in small amounts of chloroform. A thin coat of the mixtures was obtained on the walls of glass containers by slowly evaporating the solvent under a stream of nitrogen.

The residual solvent was evaporated by subjecting the sample to lyophilization. Multilamellar liposomes were then obtained by adding adequate amounts of distilled water and equilibrating the mixture at 50°C for 4 hours. To get uniform dispersions, the mixtures were subjected to vortexing intermittently.[5] The water to lipid ratio was 3 (wt/wt) in all experiments and the considered drug molar ratio R (moles of phenobarbital/moles of DSPC) ranged between R = 0 (pure DSPC) and R = .5: in fact at higher drug molar ratio a solid phenobarbital phase appeared.

### Experimental Technique

*Differential Scanning Calorimetry.* Differential Scanning Calorimetry (DSC) experiments were performed using a Perkin Elmer DSC-2C calorimeter equipped with a data processor. The scan rate was 2.5 °C/min. Sealed aluminum containers of about 20 μl capacity were used as sample holders.

### X-ray Diffraction

X ray diffraction patterns were obtained by using a vertical powder diffractometer and a low angle pinhole chamber (M. Hentchel), they both working on a rotating anode generator Rigaku Denki RU300. Ni filtered Cu-$K_\alpha$ radiation ($\lambda$ = 0.154 nm) was used.

For each molar ratio different mixtures (between 5 and 10) were made and for each mixture two samples were examined by both calorimetry and X-ray diffraction.

### Freeze-Fracture Electron Microscopy

The fracturing of rapidly frozen liposomes and the platinum-carbon replication were carried out at a temperature of $-100$°C and a pressure of $10^{-6}$ Torr in Balzer BAF301 freeze-etching apparatus, equipped with electron gun. Replicas were cleaned in methanol and the electron micrographs were taken in a CM10 Philips transmission electron microscope, operating at 80 kV.

## RESULTS

### Differential Scanning Calorimetry (DSC)

At low molar ratio of phenobarbital ($R \leq .01$) (Figure 2, middle curve, R = .001), the heating patterns show two endothermic peaks, which correspond to the $L_{\beta'}$,-$P_{\beta'}$, and $P_{\beta'}$,-$L_\alpha$ phase transitions[6] (respectively called pre transition and main transition) as in the thermograms of pure DSPC liposomes (Figure 2, lower curve). At higher concentrations, the pre transition temperature and enthalpy decrease, then at $R > .1$ they completely disappear in the most of the samples (Figure 2, upper curve and Figure 3). Moreover the main transition, which corresponds to the chain melting, occurs at lower temperature (Figures 2 and 3). The samples showing only a main transition, as reported in the upper curve of Figure 2, do not change the shape and the width at half height of the peak, with respect to the corresponding DSPC peak. The enthalpy per DSPC mole does not decrease in the considered concentration range, increasing in case around R = .1, where phase coexistence was always observed by using x-ray diffraction (see below). At R = .05 and R = .1 a widening of the main transition peak is observed.

In some cases (about 10% of all examined samples) a pre transition peak and sometimes a shoulder on the main transition peak are detected for molar ratios $R > .1$; the data of the few samples at high molar ratio, which present a shoulder on the main transition peak or a pre transition peak, are not reported in the Figure 3.

### X-ray Diffraction

The samples show the characteristic X-ray diffraction patterns of lamellar phases, at all the investigated temperatures and molar ratios; in fact in the low angle region the peak positions in $s^{-1}$ units are in the ratio 1:2:3:4 . . . .[7] The Figure 4a show the low angle diffraction patterns for different phenobarbital molar ratios observed

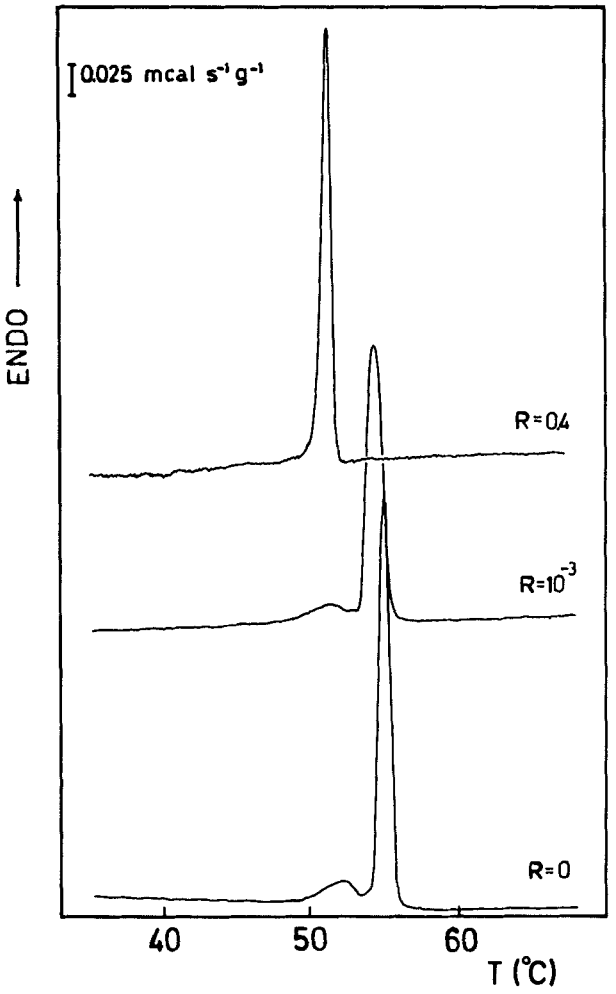



FIGURE 2 Calorimetric heating scans for R = 0 (lower curve), R = .001 (middle curve) and R = 0.4 (upper curve). Scan rate 2.5 K/min.

at room temperature (25°C). The corresponding lamellar thicknesses, determined by applying the Bragg equation, are reported in the Figure 5, as a function of the phenobarbital molar ratio.

The low angle patterns provide information on the long range organization of the phases: in particular we observe that at drug molar ratios lower than R = 0.05, the phase is a lamellar gel $L_{\beta'}$, (Figure 4a, middle curve), similar to that of pure DSPC liposomes in water excess (Figure 4a, lower curve).[7] The very intense first order peaks of the low angle patterns corresponding to R = 0 and to R = .001, are not detectable as their angular position is inside the divergence of the primary X-ray beam. Their position was then calculated from the pictures of the low angle camera.

For R > 0.1, a lamellar repetition distance is deduced (about 5.3 nm) lower than in pure DSPC (6.8 nm in $L_{\beta'}$, phase) (Figures 4 and 5). For molar ratio R between

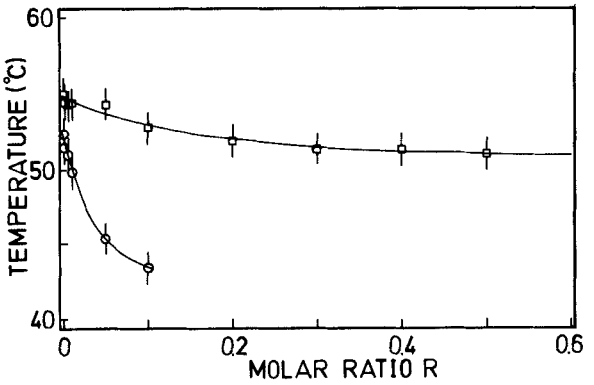



FIGURE 3 Calorimetric peak maximum temperatures vs. the phenobarbital/DSPC molar ratio "R". DSPC/water = 1/3 (w/w); squares: higher temperature peak; circles: lower temperature peak.

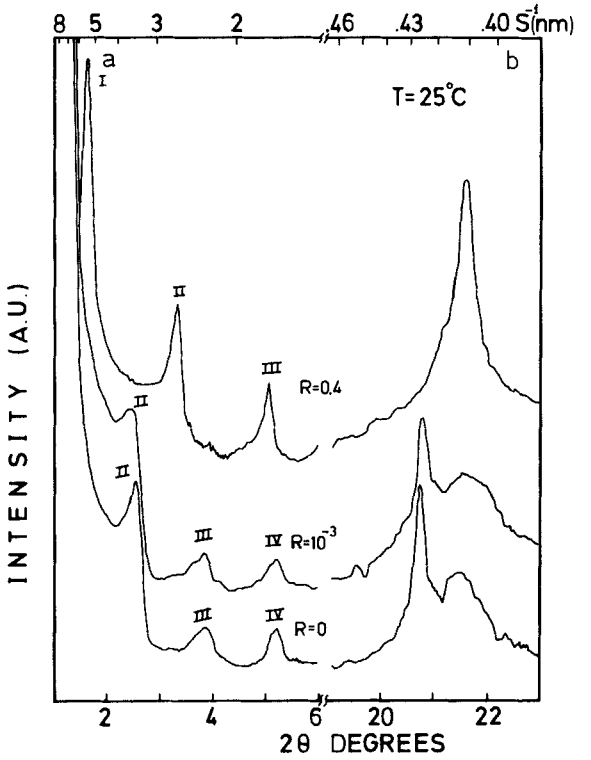



FIGURE 4 X-ray diffraction patterns from reference sample (R = 0, lower curves), from R = .001 (middle curves) and from R = .4 (upper curves), as obtained by diffractometer: (a) low angle and (b) high angle patterns. Lower scale: take off angle 2Θ(°). Higher scale: $s^{-1}$ units (nm), being $s = 2 * \sin(\Theta)/\lambda$.

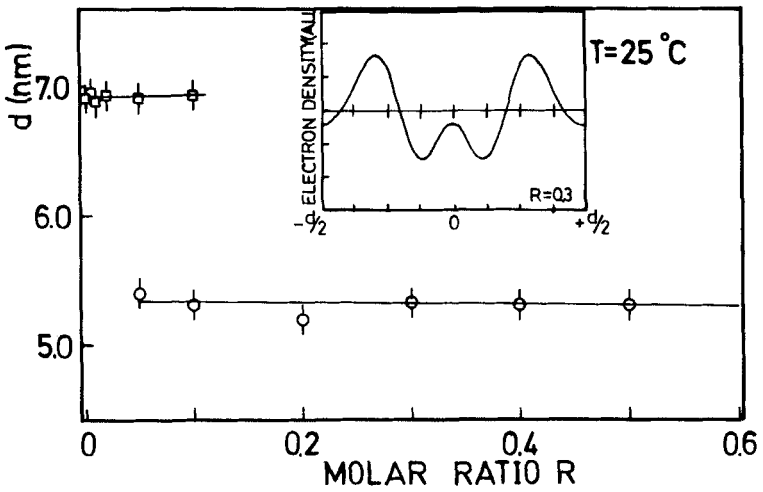



FIGURE 5 Lamellar repeat spacing "*d*", vs. molar ratio R at the temperature $T = 25°C$. Squares: $L_{\beta'}$, phase, circles: lamellar phase at lower periodicity. In the insert: proposed electron density profile of the lamellar phase at lower periodicity.

.05 and .1 two sets of peaks are detectable, corresponding to lamellar repetition distances of 6.8 and 5.3 nm, indicating that two lamellar phases coexist. Also those samples which were found to be characterized by anomalous DSC profiles at R > .1, exhibit a phase coexistence at room temperature.

The high angle diagrams, which are sensitive to the short range conformation of the chains, show, for drug molar ratios lower than .05, two closely spaced peaks, one sharp and the other more diffuse (Figure 4b, middle curve), as it occurs for the pure DSPC $L_{\beta'}$ phase (Figure 4b lower curve). This indicates a tilted chains packing in a quasi-hexagonal two-dimensional lattice. For higher molar ratios, in correspondence with the appearance of the lower lamellar repetition distance, a unique sharp peak shifted toward higher diffraction angle appears (Figure 4b, upper curve), indicating that the chains are perpendicular to the lamellar planes (β conformation) and organized in a two-dimensional hexagonal lattice.[8,9] The samples in which a phase coexistence exists are characterized in the high angle region by the presence of peaks corresponding to the two chain packings as indicated in Figure 6 (R = .05 and R = .1).

The results showed in both Figures 5 and 6 show that a phase transition occurs at $R \approx .1$: in fact the $L_{\beta'}$, phase is replaced by a new lamellar phase, characterized by the β-conformation of the chains and by a very low lamellar repeat spacing.

Also at higher temperature the X-ray diagrams depend on the drug molar ratio: for $R \leq 0.1$, above the first calorimetric peak a $P_{\beta'}$, phase is present and at temperatures above the second calorimetric peak a liquid crystalline $L_{\alpha}$ phase occurs.

For higher concentrations, the samples, having a lamellar thickness of about 5.3 nm at room temperature, do not change the phase up to the chain melting, in agreement with the disappearance of the pre-transition peak in the DSC patterns.

Concerning those samples which present a phase coexistence at room temperature, they show as a function of the temperature a progressive disappearance of

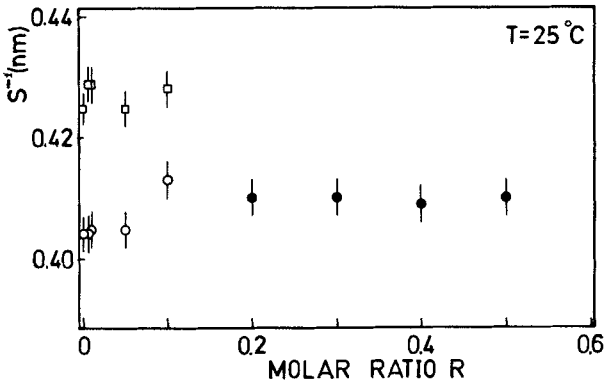



FIGURE 6 Position of the x-ray high angle peaks in $s^{-1}$ unit vs. molar ratio $R$ at room temperature. Open circles and squares: $L_{\beta'}$, phase; filled circles: lamellar phase at lower periodicity.

the lamellar phase at higher periodicity and an increasing of the peak intensities corresponding to the lower periodicity (Figure 7). Only this last periodicity is then present few degrees before the chain melting temperature. However in few cases, the $L_{\beta'}$, phase changes into a $P_{\beta'}$, phase, which coexists with the low periodicity phase up to the chain melting transition.

At temperatures higher than the chain melting temperature, the diffraction patterns show, at all investigated drug concentrations, a lamellar phase with melted chains, as indicated by the presence of the broad peak in the high angle region (insert of the Figure 8). The lamellar periodicity increases, from 6.8 to 7.1 nm, at low molar ratios and remains almost constant for higher drug concentrations (Figure 8).

### Transmission Electron Microscopy

In order to study the phase coexistence, which sometime occurs at room temperature, in the high concentration mixtures, some freeze fracture pictures were taken by transmission electron microscopy in the case of a sample (R = 0.2), characterized by a pre transition peak in DSC heating scan and by the coexistence of two phases (the $L_{\beta'}$, phase and the low periodicity phase) at room temperature in the X-ray diffraction patterns. The obtained images show two liposomes populations of different shape and an example of each population is reported in Figure 9. The different shapes observed in the two liposomes suggest that the phase separation is due to a nonhomogeneous distribution of the drug. In fact it is reasonable to assume that in the spheric liposome the drug molar ratio is lower than that in the kidney-shaped one; it is also possible that in the last liposomes a lateral phase separation occurs, similar to the one found in cholesterol-lecithin systems.[10]

## DISCUSSION

Calorimetric data (Figures 2 and 3), showing for R > .1 a small decrease in the chain melting temperature (from 54 to 51°C), suggest that the drug slightly increases

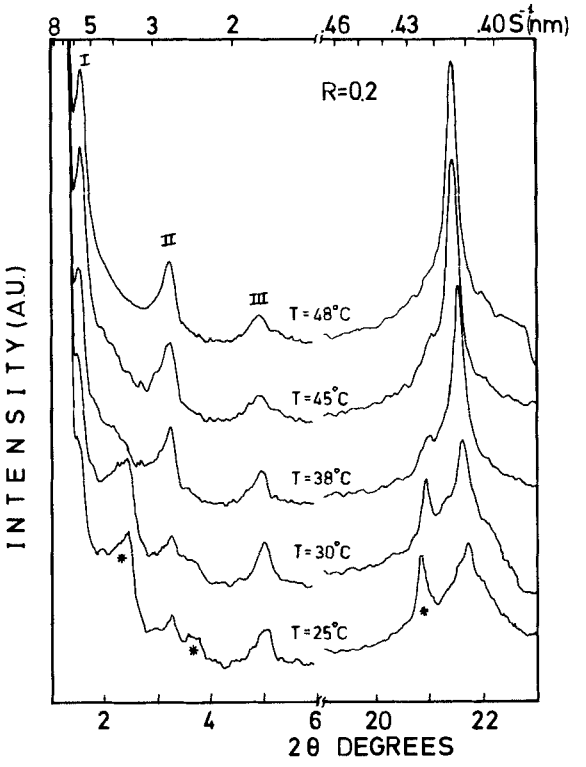



FIGURE 7 High angle X ray diffraction patterns of a sample (R = .2), which presents, at room temperature a phase coexistence. Lower scale: take off angle 2Θ(°). Higher scale: $s^{-1}$ units (nm). The peaks marked by an asterisk are related to the $L_{\beta'}$ phase.

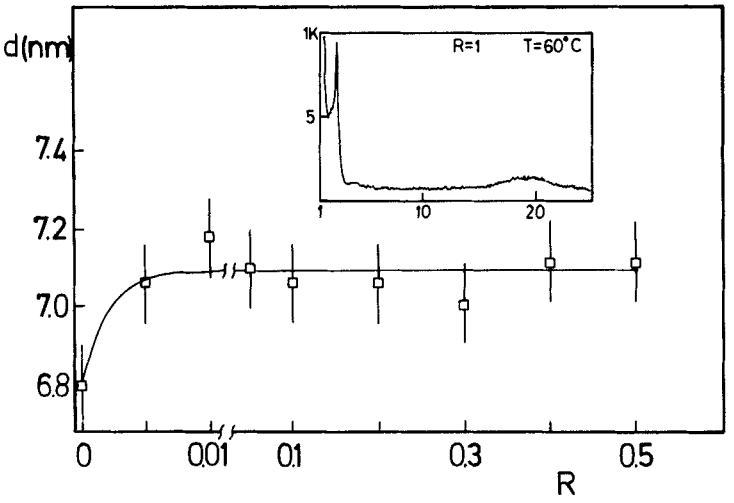
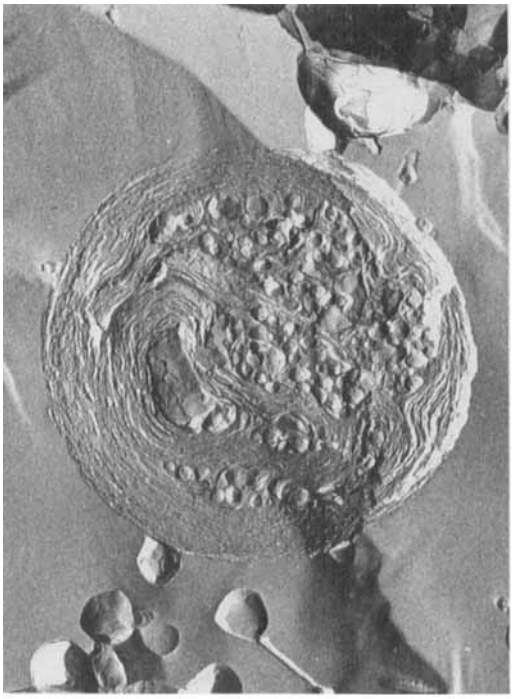



FIGURE 8 Lamellar repeat spacing "$d$," vs. molar ratio R; $T$ = 65°C. In the insert: X-ray diffraction patterns of the $L_{\alpha}$ phase, R = .2.



(a)

(b)

FIGURE 9 Electron micrographs of freeze-fracture replicas, prepared from DSPC-Phenobarbital multilamellar vescicles and quenched from room temperature. R = .2, sample with phase coexistence, (a) spheric liposome; (b) kidney shaped liposome.

the fluidity in the bilayer, as in the case of azelaic acid and DSPC mixtures,[11] while other drugs as propranolol[12] or diltiazem[13] induce a higher fluidification in DPPC liposomes. The disappearance of the pre transition, which is related to the interaction between the choline moiety and the bound water,[14] could indicate a drug localization in the polar region of the phospholipids; the lowering of the chain melting transition without enthalpy decrease suggests that the drug interacts with the head group and only partially penetrates between the chains.[15]

The X-ray diffraction patterns showed in Figure 4 indicate that the structure observed for phenobarbital molar ratio lower than 0.05, at room temperature, is a $L_{\beta'}$ phase; considering also that the measured lamellar thickness is about 6.8 nm, we estimate that this structure corresponds roughly to a unperturbed lamellar $L_{\beta'}$ phase of pure DSPC.[7] On the contrary, the lamellar structure observed at phenobarbital molar ratios higher than about 0.2 is characterized by an unique sharp peak in the high angle X-ray diffraction pattern, which suggests that the chains are stiff, extended, and perpendicular to the lamellar planes,[8,9] as in the $L_{\beta}$ phase. However, considering the loss of the chain tilt, an increase of the lamellar repeat spacing is expected, while the low angle patterns show a decrease of the lamellar repeat spacing from 6.8 to 5.3 nm.

We have obtained similar DSC and X-ray diffraction results in the case of DSPC liposomes doped by an amphiphilic drug, the dicarboxylic azelaic acid[11]; in that case the electron density profile showed an interdigitated gel phase, with rigid chain stiff and perpendicular to the lamellar planes.

A similar interdigitate lamellar phase was observed by McIntosh *et al.*[16] in the DPPC-water system after addition of charged and not charged surface-active molecules: in the former case, the structure shows an increase of the water thickness between the lipid bilayers (in excess water condition), with an interlayer distance of about 10 nm, in the latter case no increase of the water layer was observed and a layer thickness was about 5 nm (as in the case of azelaic acid and DSPC system in excess water condition[11]). In both cases the lipid thickness is about 3 nm and the electron density profile shows a density maximum due to the chain interdigitation at the centre of the bilayer.

Our experimental results, nearly identical to those obtained for the azelaic acid, suggest that also in the present case the drug molecules interact with polar head groups, slightly penetrating in the hydrocarbon region of the bilayer, increase the area per lipid molecule and, at higher molar ratio, induce a chain interdigitation.

It could be interesting to calculate the electron density profile through the bilayer to confirm this hypothesis and to eventually evaluate the position of the phenobarbital molecule in the bilayer. In order to determine the signes of the structure factors relative to the observed reflections, we tried to perform a swelling experiment series: unfortunately the phase coexistence, which in excess water condition seldom appears, always occurs at low water contents, so that the intensity analysis was not possible. Nevertheless we report here an electron density profile relative to the gel phase with lower periodicity (insert of Figure 5) observed pure in a sample in excess water, containing phenobarbital in a molar ratio R = 0.2. Such a profile has been calculated from the experimental intensities using the same signs for the structure factors as obtained in the analysis of the similar gel phase observed

in the DSPC/azelaic acid/water system.[11] In fact the X-ray profiles are very similar in both systems: in particular, the lamellar periodicities (as determined from the position of the low angle peaks) as well as the intensity ratios for the first three orders are almost the same; moreover also the high angle scattering appears quite similar in shape and in position for the two systems. However, the fourth order is absent in the DSPC/azelaic acid system: a careful inspection of the electron density profiles calculated in the present case indicates that choice of this sign is unique. In fact, a "−" sign corresponds to an electron density map not compatible with a chain interdigitation and with a lipidic sublayer thickness which is too low (≈4.2 nm) to suppose fully elongated, stiff and untilted hydrocarbon chains as indicated by the sharp high angle peak. The electron density profile corresponding to the (+ -- +) choice is then shown in the insert of Figure 5: this profile clearly indicates that the gel phase is interdigitated.

Considering also the interaction of the phenobarbital molecule with the phospholipidic head group, inferred from the calorimetric results, the interpenetration of lipid hydrocarbon chains, appears as the most probable explanation of the experimental data.

In what to concerns the phase coexistence, observed at room temperature, the electron micrographs of freeze-fractured replicas suggest that the occurrence of different calorimetry and X-ray diffraction profiles at the same drug concentrations could be explained by assuming a non-uniform drug distribution in the fully hydrated liposomes, in spite of the constant attention to the protocol of the liposome preparation, which in experiments with other drugs gave reproducible results.[10,11]

At last in the $L_\alpha$ phase, at all examined phenobarbital concentrations, the interlayer thickness is higher than in pure DSPC liposomes, suggesting that the drug molecules induce in the melted chain phase a chain stiffening, thus probably raising the orientational order, as in the case of the cholesterol-DSPC system.[17]

## CONCLUSIONS

The presence of the phenobarbital drug in the multilamellar DSPC liposomes, does not destroy the phospholipid lamellar arrangement at all molar ratios up to the saturation and, at higher molar ratios, it induces a β conformation of the chains, which thus become perpendicular to the lamellae. Moreover the calorimetric and x-ray results suggest that the phenobarbital molecule interacts with the phospholipid head groups and slightly penetrates in the hydrocarbon chain region, so that, at molar ratio higher than R = 0.1, the gel lamellar phase could be an interdigitated phase, as occurs for liposomes doped by several surface-active molecules at high concentration.[11,16]

Finally, the calorimetric results show that the drug slightly fluidizes the lipid membrane in the gel phase, while from the diffraction data it is possible to infer a lowering of the chain orientational disorder in the melted chain phase.

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