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DSC study of the action of phenylbutazone on DMPC and DPPC bilayers

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Summary

Phenylbutazone (PhB) is a powerful anti-inflammatory drug that is very sparingly soluble in water (solubility $\simeq 1 \times 10^{-3}$ M). This drug is capable of altering the phase transition temperature of phospholipid bilayers consisting of pure dimyristoyl- or dipalmitoylphosphatidylcholine without changing their calorimetric enthalpy (ΔH_{cal}), but decreasing their cooperativity unit size, as shown by differential scanning calorimetric (DSC) measurements. On interaction with PhB, multilamellar liposomes (MLV) of dimyristoylphosphatidylcholine (DMPC) undergo lateral phase separation owing to the immiscibility in the bilayer plane. In the authors' view, phenylbutazone lies preferentially in the polar region of the bilayer. In this way, the intercalated drug molecules can disrupt hydrogen bonds spanning between adjacent head-groups, thereby destroying the specific structural arrangement of a specific polar head-group region. The pH of the medium is the determining factor – through the degree of ionization of the drug and the interface – on which qualitative and quantitative changes in the main thermotropic transition of phospholipids induced by PhB depend.

Introduction

Various studies on non-steroidal anti-inflammatory drugs (NSAIDS) (Gryglewski, 1974; Hwang and Shen, 1981) have shown that, in addition to the known fact that they inhibit the biosynthesis of prostaglandins, which are essential mediators of the inflammation process, some of them have additional effects related to the plasmatic membrane.

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Surface tension experiments on phospholipid monolayers have also shown the ability of these drugs to act on them (Queraltó Moreno et al., 1984). Thus, the ability of some of these drugs to alter the phase transition undergone by phospholipids (PL) has been demonstrated by using liposomes and both differential scanning calorimetry (DSC), fluorescence quantum yield measurements and fluorescence anisotropic measurements with molecular probes such as perylene and 1,6-diphenyl-1,3,5-hexatriene (DPH) embedded in bilayers (Chantres et al., 1984, 1986). On the other hand, phenylbutazone (PhB) has been shown to induce shape changes in erythrocytes (Fujii et al., 1979), stabilize liposomal membranes

(Mizushima et al., 1975) and modify the C calorimetric transition of erythrocyte membranes (Snow et al., 1978; Davio and Low, 1982).

In addition to the above features, determining the potential use of liposomes as drug delivery systems requires elucidating the action of the encapsulating chemical on the physical status of the bilayer in order to be able to predict the stability of the particles involved and the drug-release behaviour.

On intercalation, drug molecules decrease the temperature at which phospholipid membranes undergo the transition from the gel to liquid crystalline state. This temperature is characteristic of each phospholipid and depends on the molecular structure of the fatty acid chains and the polar head-groups of the phospholipid molecules. The thermotropic behaviour of phospholipids has been investigated by using various techniques including NMR, X-ray diffraction, synchrotron radiation, fluorescence spectroscopy and FTIR spectroscopy (Cullis and De Kruijff, 1979; Tilcock et al., 1982; Hong et al., 1988; Gómez-Fernández et al., 1991; Quinn and Lis, 1991; Yin et al., 1991). On the other hand, the thermodynamic parameters for the gel to liquid crystalline phase transition of liposomes are best obtained by DSC.

This work reports on a study of the effects of PhB on the aforementioned transition, with special emphasis on the role of the ionization state of PhB on thermogram changes in terms of its relative location with respect to the bilayer.

Experimental

Materials

L-α-Dimyristoylphosphatidylcholine (DMPC) and L-α-dipalmitoylphosphatidylcholine (DPPC) were purchased from Sigma Chemical Co. Their purity was checked by thin-layer chromatography (Bangham et al., 1974). Both phospholipids were stored in chloroform solutions that were placed in a nitrogen atmosphere at −20°C. They were analysed quantitatively by the method of Bartlett (1959).

Phenylbutazone (4-butyl-1,2-diphenyl-3,5-pyrazolidinedione) was complementarily supplied by Laboratorios Lafarquim. Its purity was checked by IR spectroscopic and melting point measurements. After desiccation, the product was dissolved in methanol and stored in the dark at −20°C.

The buffer solutions used included HOAc-NaOAc of pH 4.5, Tris-HCl of pH 7.0 and $NaHCO_3/Na_2CO_3$ of pH 10.0. All reagents used were analytical-grade chemicals and bidistilled water was employed throughout.

Methods

Preparation of the liposomal dispersions

We chose to use multilamellar liposomes (MLV), which allow for much more reproducible calorimetric measurements than small unilamellar liposomes (SUV) (Steim, 1968; Suurkuus et al., 1976; Kantor et al., 1977) and feature a higher transition cooperativity than both SUV and LUV (large unilamellar liposomes), therefore, being more suitable for studying the effects of external agents on the bilayer by the DSC technique (Mabrey and Sturtevant, 1976).

The liposomal dispersions were prepared essentially according to previously reported procedures (Chantres et al., 1986). The overall sample volume and phospholipid concentration used were 100 μl and 70 mM, respectively. Reproducibility of the results was ensured by allowing the DMPC and DPPC dispersions to stand at room temperature for 2 and 3 h, respectively, after preparation. PhB was added during the liposomal preparation procedure at the phospholipid film formation stage.

Calorimetric measurements

The study was performed with a Mettler TA 3000 thermal analyser equipped with a DSC-20 measuring cell. Thermometric and enthalpic calibration was carried out with indium, zinc and lead. A second, fine calibration of the thermometric scale was performed by using water, palmitic acid and indium. The calibration results were consistent with the melting points and fu-

sion enthalpies of standards of saturated fatty acids containing between 10 and 16 carbon atoms.

Samples volumes of 20 μl containing approx. 1 mg of phospholipid were placed in 50-μl crucibles and scanned at a rate of 2 K min^{-1}. Before the temperature programme was executed, samples were kept at the initial temperature (5°C for DMPC and 15°C for DPPC) for 15 min. The reproducibility of the rising and falling temperature scans was quite satisfactory, particularly for the fairly high rates at which they were performed. However, this did reflect in the usual hysteresis between the two types of scan.

The average size of the transition cooperative unit, $\langle \nu \rangle$, was calculated from the measured calorimetric enthalpy, ΔH_{cal}, by using the equation

$$\langle \nu \rangle = \frac{6.9}{\Delta H_{cal}} \cdot \frac{T_m^2}{\Delta T_{1/2}} \qquad (1)$$

where T_m is the main transition temperature and $\Delta T_{1/2}$ denotes the peak half-width at half-height (Hinz and Sturtevant, 1972).

Results

Phenylbutazone alters the thermograms obtained for the gel-liquid crystal transition undergone by DMPC and DPPC bilayers. Inasmuch as it is a weak acid ($pK_a \simeq 4.5$), the pH of the medium conditions its action as regards the position of the main transition temperature (T_m) and the degree of cooperativity of the transition.

Fig. 1 shows some of the thermograms obtained in the rising temperature scans. In all cases – even at the lowest assayed concentration – the drug caused the characteristic pre-transition of the phospholipid to disappear. One other common feature is the virtual constancy of the transition enthalpy, irrespective of the PhB concentration and pH of the medium (Fig. 2).

As can be seen in the DMPC recordings obtained at pH 4.5, the onset transition temperature (T_0) and that of the maximum (T_m) shifted gradually to lower values; also, the transition width, i.e., the temperature range spanned by the transition, increased with increase in the PhB concentration. The effects observed at pH 7.0

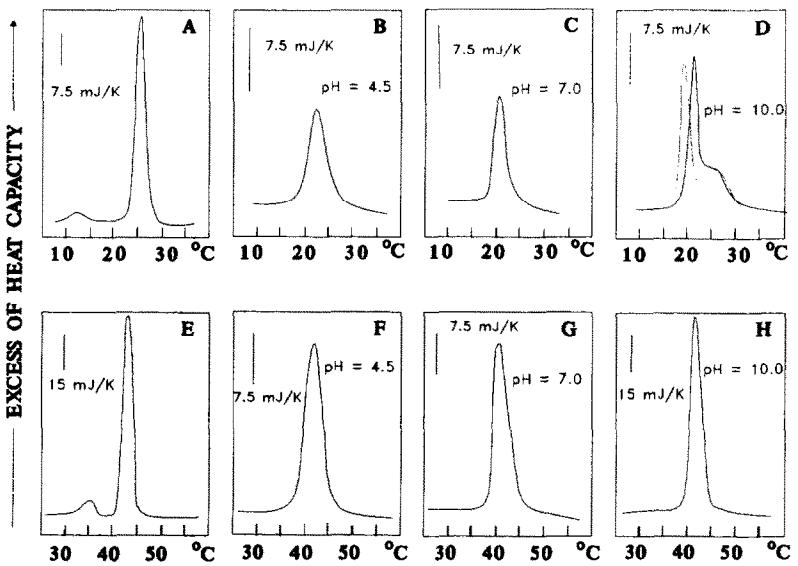

Fig. 1. DSC thermograms for the thermotropic transition of multilamellar liposomes of DMPC (A–D) and DPPC (E–H) obtained at a scan rate of 2 K min^{-1} in the absence (A and E) and presence of PhB in a mole ratio of 20% at three different pH values. The broken line (thermogram D) corresponds to a falling temperature scan, whereas the others correspond to rising temperature scans.

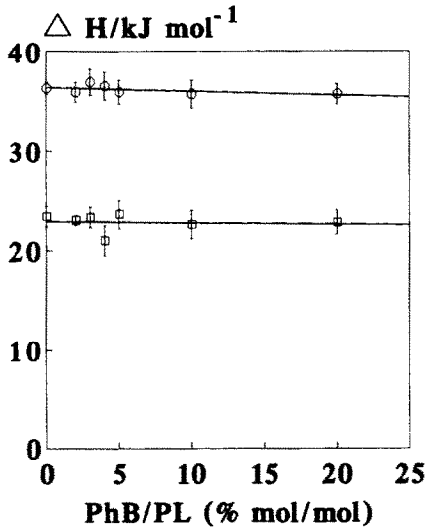

Fig. 2. Variation of the enthalpy of the main thermotropic transition of multilamellar liposomes of DMPC (□) and DPPC (○) as a function of the PhB/PL ratio at pH 4.5. Bars represent the standard deviation of the measurements on each of three liposomal samples after at least four rising temperature scans. The results obtained at pH 7.0 and 10.0 were not significantly different from those found at pH 4.5.

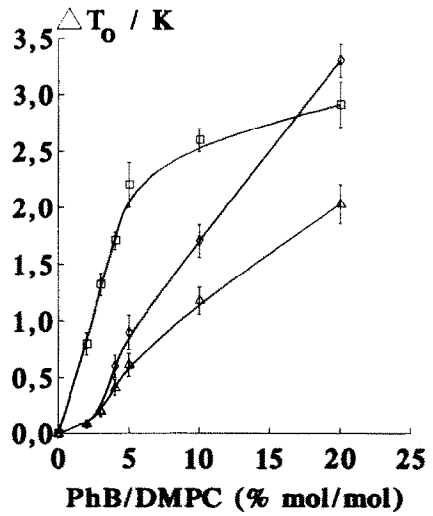

Fig. 3. Variation of the onset transition temperature change ΔT_0, as a function of the PhB/DMPC mole ratio at pH 4.5 (□), pH 7.0 (◇) and pH 10.0 (△). Bars represent the standard deviations of the measurements.

were qualitatively identical, though quantitatively smaller. On the other hand, while the decrease in T_0 and T_m was significant, the peak width ($\Delta T_{1/2}$) did not vary appreciably at pH 10.0; however, a shoulder suggesting the occurrence of phase separation in the bilayer was clearly observed at this pH and a PhB mole ratio of 20%.

The DPP-PhB system behaves similarly to DMPC. Qualitatively, the effect of phenylbutazone on the phase transition of DPPC is subject to the same concentration and pH dependence as for DMPC. However, there are significant quantitative differences. Thus, at any pH, the decrease in T_0 and T_m is less marked, as is the increase in the peak width ($\Delta T_{1/2}$).

The thermograms obtained in the falling temperature scans (not shown here) were similar to their rising temperature counterparts, reflecting their characteristic hysteresis and no pre-transition – not even for the pure phospholipid samples. On the other hand, the shoulder observed at pH 10.0 and a 20% PhB/DMPC mole ratio appeared in the form of a second peak with a T_m value very similar to that of the pure phospholipid (PL) in the falling temperature scan (Fig. 1D). In fact, the scans performed at high PhB/PL mole ratios reflect the potential occurrence of multiple transitions at the pH values studied.

Figs 3 and 4 show the fall in T_c, ΔT_c, as function of the PhB/PL ratio for both systems at

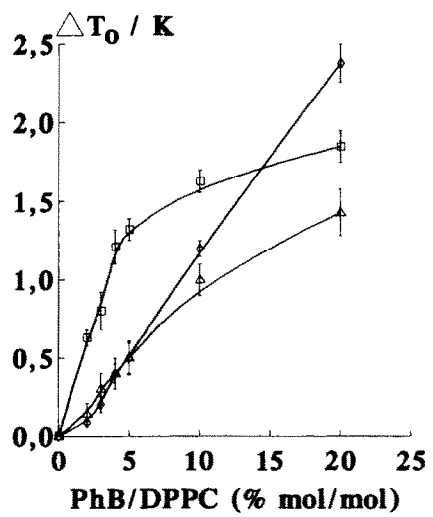

Fig. 4. Variation of the onset transition temperature change ΔT_c, as a function of the PhB/DPPC mole ratio at pH 4.5 (□), pH 7.0 (◇), and pH 10.0 (△). Bars represent the standard deviations of the measurements.

the three pH values assayed. The similarity between the two graphs is outstanding. Both seem to reflect a saturation phenomenon at pH 4.5 which is not observed at any of the PhB/PL ratios assayed at the other two pH values. On the other hand, while the drug activity is clearly observed at a 2% mole ratio at pH 4.5, there seems to be an 'induction interval' prior to ΔT_c taking a significant value at pH 7.0 and 10.0.

Fig. 5 shows the effects on the transition width arising from changes in the cooperative unit size, whose variation with the PhB/PL ratio is also depicted in the graph. The qualitative effect on both phospholipids is quite similar and is maximal at pH 4.5 and minimal at pH 10.0. The activity is higher on DMPC bilayers than on DPPC bilayers. Thus, for a phenylbutazone mole ratio of 20%, the average cooperative unit size decreases from 210 to 70 for the DMPC bilayers and from 113 to 55 for the DPPC bilayers. The concentration threshold requisite is clearly observed at pH 7.0 and 10.0 for the DMPC liposomes. Also, both systems are subject to a saturation effect at pH 4.5 and 10.0.

Discussion

Even though cell membranes are believed to be in a highly 'fluid' state, because of the high proportion of unsaturated fatty acid chains that make up phospholipids, bilayers are not in such a state throughout; rather, the above-mentioned fluid regions co-exist with more 'rigid' regions in the context of a functional strategy. This calls for a serious study if the activity of various agents capable of altering the physical state of these domains is to be determined. On the other hand,

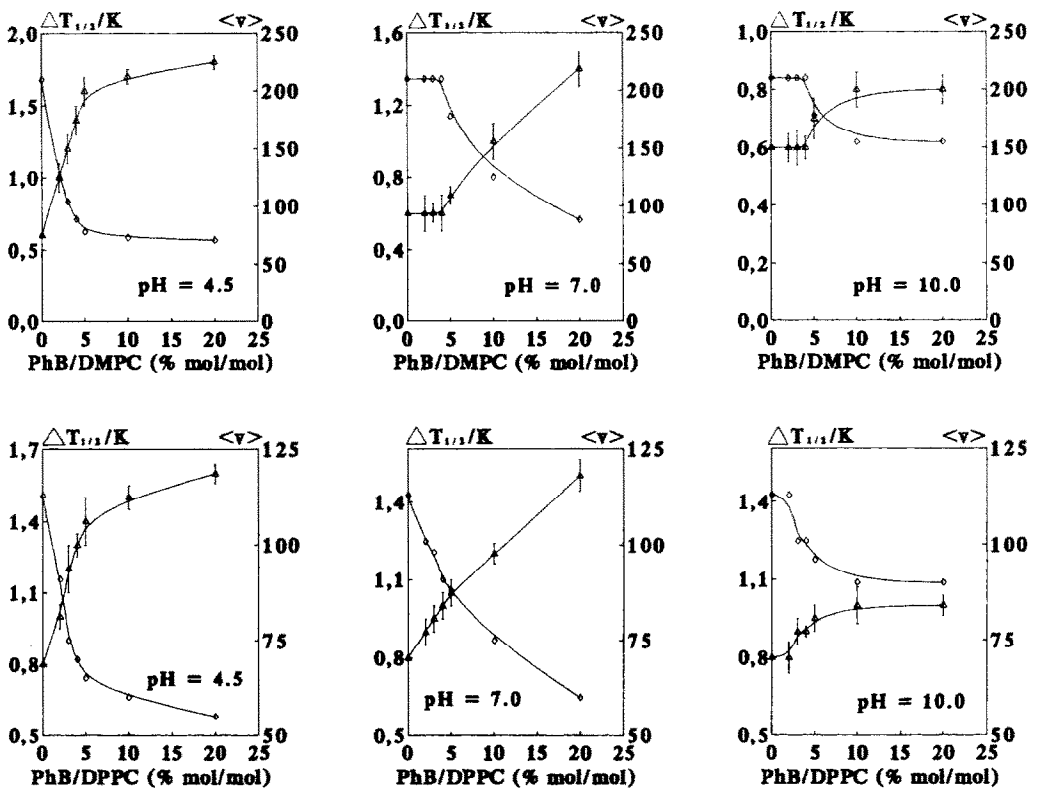

Fig. 5. Variation of the transition width $\Delta T_{1/2}$ (△) and the average cooperative unit size calculated from it $\langle \nu \rangle$ (Hinz and Sturtevant, 1972) (◇) as a function of the PhB/PL mole ratio at the three pH values assayed. Bars represent the standard deviations of the $\Delta T_{1/2}$ measurements.

the growing use of liposomes as drug delivery systems requires the potential alterations to those features of the bilayer that may affect its stability and barrier properties to be studied in depth in order to improve their bioavailability and therapeutic index.

Below the phase-transition temperature, the PL molecules in the gel phase are packed in a highly ordered manner with the hydrocarbon chains of the fatty acids in an all-*trans* conformation that hinders motional freedom considerably. At the transition to the liquid crystalline state, the hydrocarbon chains gain more motional freedom, some C-C bonds adopt a *gauche* conformation, the bilayer thickness increases and the bilayer expands as a result. In addition, packing in the region of the polar head-groups is believed to be loosened.

Our experimental results confirm the ability of PhB to alter the thermotropic phase transition of multilamellar vesicles of DMPC and DPPC. The pre-transition of phosphatidylcholines, which is ascribed to the transition from an L_β structure to a P_β structure, is highly sensitive to the presence of other molecules capable of exerting an action from the polar group region, even in very low proportions (Ladbrooke et al., 1968; Cater et al., 1974; Chapman et al., 1974; Hwang and Shen, 1981); any alterations in this respect will therefore be scarcely specific.

As far as the ability of PhB to alter the main phase transition is concerned, it is markedly influenced by the pH of the medium – with the exception of the enthalpy change – on account of its weakly acidic character ($pK_a \simeq 4.5$). In the phospholipid, the apparent pK_a values at the liposomal surface are 3.7 and 11.6 for the phosphate and trimethylammonium group, respectively (Seimiya and Ohki, 1973), so the fraction of the zwitterionic form is approx. 97.6% at pH 10.0, 99.9% at pH 7.0 and 86.3% at pH 4.5. Hence, the first difficulty involved in the incorporation of 100% ionized PhB into the bilayer at pH 10.0 lies in the electrostatic barrier posed by the prevalent phosphate group. This effect is somewhat less marked at pH 7.0; moreover, the degree of ionization of PhB at this pH is such that there is some undissociated species (approx. 4.4×10^{-5} M for a 20% PhB/PL mole ratio according to our experiments) in the medium. Such an undissociated concentration can not be considered to be negligible in terms of its potential contribution to the observed effects; in fact, being a neutral species, its partitioning equilibrium between the aqueous and lipid phase will be displaced to the latter. However, the differences encountered at pH 7.0 and 10.0 are only apparent at high drug concentrations. On the other hand, at pH 4.5 the neutral form of PhB accounts for less than 50%, so the drug is more liable to be incorporated into the bilayer and induce greater perturbations.

The significant differences between the effect of PhB on the two phospholipids can be ascribed to the following facts: inasmuch as DPPC has two

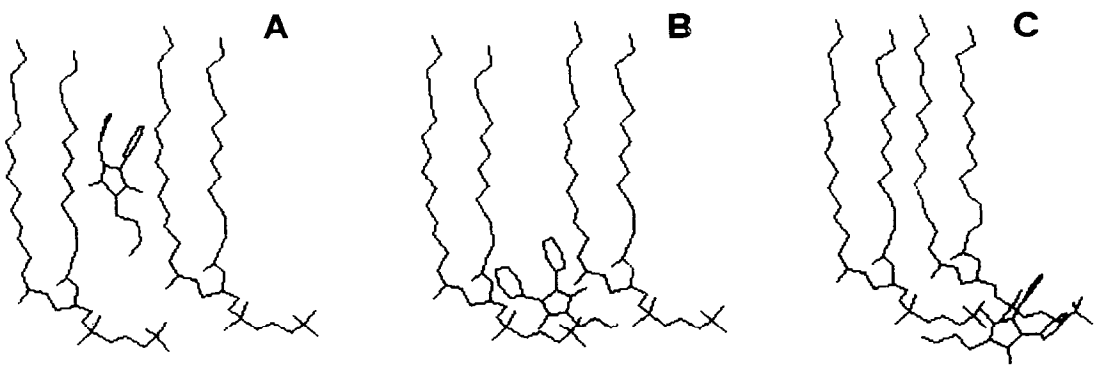

Fig. 6. Scheme showing three possible locations of the phenylbutazone molecule with respect to a DMPC bilayer. (A) Deeply inside the bilayer, which would result in major alterations to the phase transition. (B) Situation where PhB would lie at the same level as glycerol, from which it could disturb the interfacial region and the phospholipid chain packing. (C) At an outer level, where the drug would only interact through polar groups and would hardly disturb the acyclic chain packing.

extra methylene groups, van der Waals interactions will be stronger, so any packing loosening caused by an external agent will affect this phospholipid less markedly than it will DMPC. As a result, the transition parameters will be affected to a lesser extent.

The cooperative unit size is highly sensitive to the presence of external agents. On the other hand, the alterations induced by PhB result in no dramatic changes in the calorimetric profiles that might be consistent with major alterations to the bilayer (e.g., in the situation of PhB in scheme A or B, Fig. 6), not even at the highest concentrations assayed. Accordingly, the PhB-PL interaction may conform to one of the models reported by Verteuil et al. (1981). Such a model describes the temperature dependence of the relationships between lipid bilayers and small molecules and takes account of both Coulomb and van der Waals interactions. It is an interstitial model according to which the perturbation takes place in the vicinity of the polar groups (see the scheme in Fig. 6C). The model also takes account of direct interactions between adjacent molecules and the external agent. One of the predictions of this model is the occurrence of a phase that is rich in the agent; this lies in interstices and thus 'fluidizes' the neighbouring phospholipid chains, which have a lower transition temperature as a result. This situation can occur in a state of immiscibility in the bilayer plane that gives rise to lateral separation of the phase, as shown in our thermograms obtained at pH 10.0 and, in general, in all the experiments carried out at a high PhB/PL ratio.

We therefore believe that phenylbutazone lies preferentially in the polar region and interacts with the polar groups of phosphatidylcholine, scarcely penetrating into the interfacial region delimited by glycerol skeleton and esters bounds of the acyclic chains. In this way, intercalated drug molecules can disrupt hydrogen bonds spanning between adjacent head-groups, thereby destroying the specific structural arrangement of an also specific polar head-group region. The pH of the medium would be the one factor determining the degree of penetration of phenylbutazone through the degree of ionization of the drug and the interface (proportion of the zwitterionic form of the phospholipid). Qualitative and quantitative alterations to the main thermotropic transition of the phospholipid would thus depend on such a factor.

Acknowledgment

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References

Bangham, A.D., Hill, M.W. and Miller, N.G.A., Preparation and use of liposomes as models in biological membranes. *Methods Membrane Biol.*, 1 (1974) 1–68.

Bartlett, G.R., Phosphorus assay in column in chromatography. *J. Biol. Chem.*, 234 (1959) 466–468.

Cater, B.A., Chapman, D., Hawes, S. and Saville, J., Lipid phase transitions and drug interactions. *Biochim. Biophys. Acta*, 363 (1974) 54–69.

Chantres, J.R., Sainz, M.C. and Ponce-Garcia, J.M., Accion de indometacina y dexametasona sobre la transicion de fase de bicapas lipidicas. *An. Real Acad. Farm.*, 50 (1984) 581–598.

Chantres, J.R., Sainz, M.C., Ponce-Garcia, J.M. and Otero Aenlle, E., Modificacion de los parametros de transicion de fase y de orientacion en bicapas de fosfolipidos por el antiinflamatorio sulfuro de sulindac. *An. Quim.*, 82 (1986) 185–193.

Chapman, D., Urbina, J. and Keough, K.M., Calorimetric studies of dilute aqueous suspensions of bilayers formed from synthetic L-α-lecithins. *J. Biol. Chem.*, 249 (1974) 2512–2520.

Cullis, P.R. and De Kruijff, B., Lipid polymorphism and the functional roles of lipids in biological membranes. *Biochim. Biophys. Acta*, 559 (1979) 31–42.

Davio, S.R. and Low, P.S., Characterization of the calorimetric C transition of the human erythrocyte. *Biochemistry*, 21 (1982) 3585–3593.

Fujii, T., Sato, T., Tamura, A., Watatsuki, M. and Kanaho, Y., Saphe changes of human erythrocytes induced by various amphiphatic drugs acting on the membrane of the intact cells. *Biochem. Pharmacol.*, 28 (1979) 613–620.

Gómez-Fernández, J.C., Aranda, F.J. and Villalain, J., Location and dynamics of α-tocopherol in membranes. In Gómez-Fernández, J.C., Chapman, D. and Packer, L. (Eds), *Progress in Membrane Biotechnology*, Birkhauser, Basel, 1991, pp. 98–117.

Gryglewski, R.J., Structure-activity relationship of some prostaglandin synthetase inhibition. In Robinson, H.J. and Vane, R. (Eds), *Prostaglandin Synthetase Inhibitors*, Raven, New York, 1974, pp. 33–50.

Hinz, H.J. and Sturtevant, J.M., Calorimetric studies of dilute aqueous suspensions of bilayers formed from synthetic L-α-lecithins. *J. Biol. Chem.*, 247 (1972) 6071–6075.

Hong, K., Balwind, P.A., Allen, T.M. and Papahadjopoulos, D., Fluorometric detection of the bilayer-to-hexagonal phase transition in liposomes. *Biochemistry*, 27 (1988) 3947–3955.

Hwang, S.B. and Shen, T.Y., Membrane effects of antiinflamatory agents. 2. Interaction of nonsteroidal antiinflammatory drugs with liposomes and purple membranes. *J. Med. Chem.*, 24 (1981) 1202–1211.

Kantor, H.L., Mabrey, S., Prestegard, J.H. and Sturtevant, J.M., A calorimetric examination of stable and fusing lipid bilayer vesicles. *Biochim. Biophys. Acta*, 466 (1977) 402–410.

Ladbrooke, B.D., Williams, R.M. and Chapman, D., Studies of lecithin-cholesterol-water interactions by differential scanning calorimetry and X-ray diffraction. *Biochim. Biophys. Acta*, 150 (1968) 333–342.

Mabrey, S. and Sturtevant, J.M., Investigation of phase transitions of lipids and lipid mixtures by high sensitivity differential scanning calorimetry. *Proc. Nat. Acad. Sci. USA*, 73 (1976) 3862–3866.

Mizushima, Y., Ishiiy, Y. and Masumoto, S., Physico-chemical properties of potent nonsteroidal anti-inflammatory drugs. *Biochem. Pharmacol.*, 24 (1975) 1589–1592.

Queraltó Moreno, A., Castro Ruiz, R.M. and Cordoba Gallardo, J., Estudio en monocapas del sistema piketoprofen-dimiristil lecitina. *An. Real Acad. Farm.*, 50 (1984) 187–193.

Quinn, P.J. and Lis, L.J., Dynamic X-ray diffraction studies of phase transitions in lipid-water systems. In Gómez-Fernández, J.C., Chapman, D. and Packer, L. (Eds), *Progress in Membrane Biotechnology*, Birkhauser, Basel, 1991, pp. 12–29.

Seimiya, T. and Ohki, S., Ionic structure of phospholipid membranes, and binding of calcium ions. *Biochim. Biophys. Acta*, 298 (1973) 546–558.

Snow, J.W., Brandts, J.F. and Low, P.S., The effects of anion transport inhibitors of structural transitions in erythrocyte membranes. *Biochim. Biophys. Acta*, 512 (1978) 579–590.

Steim, J.M., Spectroscopic and calorimetric studies of biological membrane structure. In Gould, R.F. (Ed.), *Molecular Association in Biological and Related Systems*, Am. Chem. Soc., Washington, DC, 1968, pp. 259–302.

Suurkuus, J., Lentz, B.R., Barenholtz, Y. Biltonen, R.L. and Thompson, T.E., A calorimetric and fluorescent probe study of the gel-liquid crystalline phase transition in small, single-lamellar dipalmitoylphosphatidylcholine vesicles. *Biochemistry*, 15 (1976) 1393–1400.

Tilcock, C.P.S., Bally, M.B., Farren, S.B. and Cullis, P.R., Influence of cholesterol on the structural preferences of dioleoyl phosphatidylethanolamine-dioleoyl phosphatidylcholine systems: A phosphorus-31 and deuterium nuclear magnetic resonance study. *Biochemistry*, 21 (1982) 4596–4601.

Verteuil, F., Pink, D.A., Vadas, E.B. and Zuckermann, M.J., Phase diagrams for impure lipid systems applications to lipid/anaesthetic mixtures. *Biochim. Biophys. Acta*, 640 (1981) 207–222.

Yin, C.C., Lin, B.Z. and Hauser, H., Phase behavior of phosphatidic acid. In Gómez-Fernández, J.C., Chapman, D. and Packer, L. (Eds), *Progress in Membrane Biotechnology*, Birkhauser, Basel, 1991, pp. 30–39.