International Journal of Pharmaceutics, 49 (1989) 39–45
Elsevier

IJP 01641

An electron spin resonance study of skin penetration enhancers

C.L. Gay [1], T.M. Murphy [1], J. Hadgraft [1], I.W. Kellaway [1], J.C. Evans [2] and C.C. Rowlands [2]

[1] *The Welsh School of Pharmacy and* [2] *The Department of Chemistry, University of Wales, Cardiff (U.K.)*

(Received 12 May 1988)
(Accepted 14 June 1988)

Key words: Electron spin resonance; Skin penetration enhancer; Phospholipid bilayer; Stratum corneum

Summary

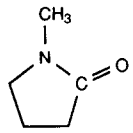
The structured intercellular lipids of the stratum corneum are thought to play an important role in controlling the transport of drugs across the skin. In this study, multilamellar dipalmitoylphosphatidylcholine liposomes were used as a model of structured lipids. The effect of temperature and skin penetration enhancers on the lipid bilayer, was assessed using a spin labelling technique, in which 5-doxyl stearic acid was incorporated into the bilayer. The order parameter (S) of the spin label was calculated from the observed spectra. An increase in temperature decreased the order parameter. This decrease is due to increased rotational movement of the acyl chain of the spin label about its long molecular axis. It indicates a decrease in order within the bilayer structure, as a result of increased flexibility of the lipid molecules. Known skin penetration enhancers, Brij 36T, *n*-decylmethyl sulphoxide and oleic acid, also decreased the order parameter of the spin label. The penetration enhancer *N*-methyl-2-pyrrolidone had no significant effect. The results obtained were confirmed using a light scattering technique, in which the phase transition temperature of the liposomes was monitored in the presence and absence of the enhancers. 5-Doxyl stearic acid was incorporated into stratum corneum, separated from human cadaver skin. The spectra obtained were similar to those of the liposomal samples and the application of *n*-decylmethyl sulphoxide had a similar effect on the order parameter.

Introduction

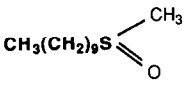
Electron spin resonance has become a valuable tool in the study of biological membranes. The spin-labelling technique involves the incorporation of a probe molecule, usually a nitroxide free radical, into the system under investigation. Analysis of the ESR spectrum of the probe yields information about the motional character of the probe in that particular environment (Hubbell and McConnell, 1969). This in turn is assumed to reflect the type and rate of molecular motion of the molecules comprising the membrane. A variety of naturally occurring and synthetic membranes have been used, but to overcome the problems associated with biological heterogeneity, many studies have employed the use of model membranes.

The percutaneous absorption of drugs is a complex process which has not yet been fully elucidated. It is, however, widely accepted, that the permeability of the skin is controlled mainly by passive diffusion through the stratum corneum, the uppermost non-viable layer of the epidermis. The keratinized cells of the stratum corneum are separated by intercellular lipids, which are structured into multilamellar bilayers. The physical and chemical properties of these lipids are thought to be of major importance in maintaining the barrier

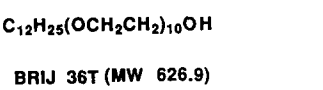
Correspondence: J. Hadgraft, The Welsh School of Pharmacy, University of Wales, Cardiff CF1 3XF, U.K.



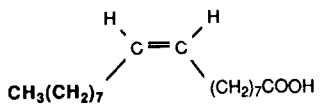
N-METHYL PYRROLIDONE (MW 99.1)



n-DECYLMETHYL SULPHOXIDE (MW 204.4)



BRIJ 36T (MW 626.9)



OLEIC ACID (MW 282.5)

Fig. 1. Molecular weight and structure of the penetration enhancers.

function of the skin (Elias, 1983). In this study dipalmitoylphosphatidylcholine (DPPC) liposomes were used as a model of structured lipids. 5-Doxyl stearic acid (5-DS) was used as the probe. In the lipid bilayer this fatty acid nitroxide molecule, orientates so that its acyl chain is perpendicular to the bilayer surface and it undergoes anisotropic motion about its long molecular axis (Hubbell and McConnell, 1969, 1971). Into this model system the penetration enhancers, polyoxyethylene 10 lauryl ether (Brij 36T), *n*-decylmethyl sulphoxide (DecMSO), *N*-methyl-2-pyrrolidone (NM2P) and oleic acid, were incorporated (the molecular structures are shown in Fig. 1). These agents have been shown to decrease the resistance of the human skin to the penetration of certain chemical species (Ashton, 1987; Barry, 1987; Touitou and Abed, 1985). Their precise mechanism of action remains unclear. We investigated the effect these agents have on DPPC lipid bilayers.

Experimental

Materials

DPPC was purchased from Sigma (Poole, U.K.). Oleic acid and 5-DS were purchased from Aldrich (Gillingham, U.K.). NM2P was purchased from BDH (Poole, U.K.). DecMSO was purchased from Cyclo products (Los Angeles, U.S.A.). Brij 36T was donated by Fisons (Loughborough, U.K.). Other reagents used were of Analar grade. All materials and reagents were used without further purification.

Preparation of DPPC liposomes for ESR spectroscopy

Multilamellar DPPC liposomes were prepared using a slightly modified form of the method of Bangham et al. (1965). In order to minimize the amount of perturbation of the bilayer caused by the incorporation of the probe, a very low concentration was used and the μmol ratio of DPPC:5-DS was kept constant at 400 : 1.

Thin lipid films containing 10 μmol DPPC, 0.025 μmol 5-DS and varied amounts of the enhancers, were formed on the inner surface of round-bottom flasks by rotary evaporation of chloroform mixtures. The films were left overnight under vacuum to remove residual solvent, after which they were hydrated with 1 ml of pH 9.6 borate buffer, at 60 °C; a temperature well above the phase transition temperature (T_c) of the pure lipid (41 °C). A high pH was chosen to ensure that all the carboxyl groups of the spin label were ionized in the phospholipid membrane (Sanson et al., 1976; Egret-Charlier et al., 1978). The samples were vigorously hand-shaken at 60 °C for approx. 2 min until liposomal dispersions had formed. This was followed by a further 10–15 min of intermittent shaking to ensure thorough mixing of the components. The samples were then cooled to room temperature, briefly centrifuged and the supernatants removed. The liposomal pellets were resuspended in 60 μl of fresh buffer and transferred to glass capillary tubes. ESR spectra were

recorded on a Varian E3 spectrometer equipped with a nitrogen flow temperature regulatory system.

To determine whether any effect of the enhancers was concentration dependent and to observe the effect of the enhancers below and above the T_c of the lipid, two sets of experiments were carried out.

(a) The concentration of each of the enhancers was varied and the temperature kept constant at 20 °C. (The concentrations expressed as percentage w/w DPPC.)
(b) The temperature was varied between 20 and 55 °C and the concentrations of the enhancers kept constant at an optimum level, determined by the previous set of experiments.

Preparation of human stratum corneum for ESR spectroscopy

Human stratum corneum was separated from whole skin using the method of Kligman and Christophers (1963). Post-mortem abdominal skin was immersed in a water bath at 60 °C for 3 min, after which the epidermis was peeled away using forceps. The epidermis was placed corneum side up, on filter papers floating on an aqueous solution of 0.0001% trypsin in 0.5% sodium bicarbonate and incubated at 37 °C for 20 h. After incubation the softened viable part of the epidermis was carefully removed using moistened cotton wool.

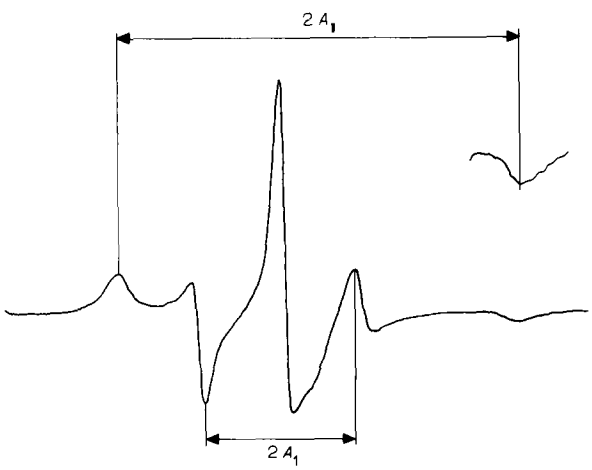


Fig. 2. Typical ESR spectrum of 5-DS in DPPC liposomes. S was determined from the indicated splittings using Eqn. 1.

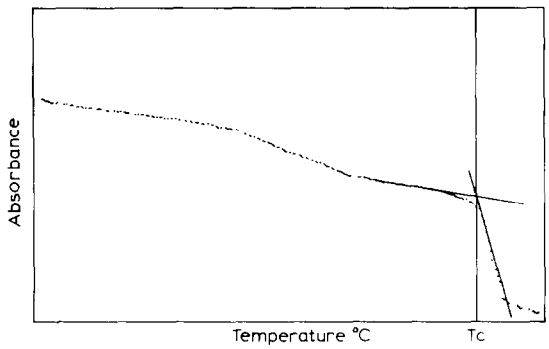


Fig. 3. Typical trace of absorbance vs temperature for DPPC liposomes. The T_c is characterised by a sudden decrease in absorbance and is determined from the change in the slope of the line as shown.

DecMSO and/or 5-DS was solvent deposited onto the stratum corneum in a minimum quantity of ethanol. The weight ratio of stratum corneum : DecMSO : 5-DS was approx. 700 : 7 : 1. The solvent was allowed to evaporate and after at least 2 h equilibration time, the sample was loaded into a quartz ESR tube and the spectrum recorded.

Order parameter measurements from ESR spectra

The order parameter (S) is a relative index of the motional character or disorder of the membrane. It gives a measure of the distribution of angles made by the hydrocarbon chains of the spin label, relative to a reference axis. This reference axis is usually chosen to be normal to the membrane surface. S has values ranging from 0 to 1 and is defined by:

$$S = \frac{A_{\parallel} - A_{\perp}}{A_{zz} - A_{xx}} \quad (1)$$

where $A_{\parallel}$ and $A_{\perp}$ are the parallel and perpendicular components of the hyperfine splittings, and A_{zz} and A_{xx} are the motional averages of the nitroxide hyperfine tensors in the directions parallel and perpendicular to the long molecular axis; their values are assumed to be 30.8 and 5.8 gauss, respectively (Hubbell and McConnell, 1971).

The order parameter was calculated from each spectrum using Eqn. 1 (see Fig. 2). A decrease in

the value of S indicated an increase in the motional freedom of the spin label.

Determination of the phase transition temperature of the liposomal suspensions

At approx. 41 °C, DPPC liposomes undergo a phase transition from a gel phase to a liquid crystalline phase. This transition is accompanied by a decrease in optical density which can be monitored using a light scattering technique (Inoue et al., 1986).

Liposomes were prepared in a similar manner to those prepared for ESR spectroscopy. Lipid films containing 1 mg of DPPC and varied amounts of the enhancers were formed on the inner surface of round-bottom flasks. The films were hydrated with 1 ml of deionised water at 60 °C and shaken as before to produce multilamellar liposomes.

The optical density of the liposomal suspensions was recorded as a function of temperature, using a modified LKB spectrophotometer, set at 500 nm. Each sample was heated at a rate of 5–10 °C/min in a water-jacketed cuvette-holder and continuously stirred with a magnetic flea. A RS590 semi-conductor temperature sensor was used to monitor the sample temperature. The absorbance vs temperature data was recorded on a BBC model B microcomputer and the traces displayed on a Novex visual display unit. The T_c for the liposomal suspension, was determined from the points of intersection as shown in Fig. 3.

Results

All the observed spectra were typical of a fatty acid spin label undergoing anisotropic motion within an ordered structure. Fig. 4a shows a typical ESR spectrum for 5-DS in DPPC liposomes at 20 °C. The broad peaks and the nature of their splitting suggest that the probe was strongly immobilized within the lipid bilayer. An increase in temperature or the inclusion of a penetration enhancer, such as DecMSO (Fig. 4b), appeared to decrease the order within the system and thus increase the rotational movement of the acyl chain of the spin label, about its long molecular axis.

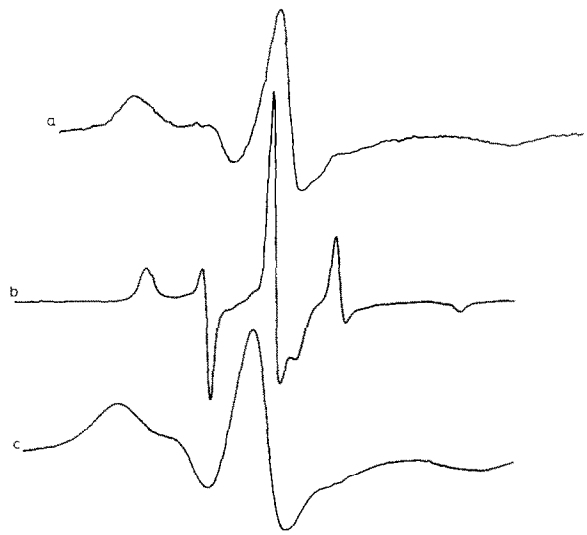


Fig. 4. ESR spectra of 5-DS at 20 °C. (a) DPPC liposomes; (b) DPPC liposomes treated with DecMSO; (c) stratum corneum.

The peaks became narrow and more pronounced and the parallel component of the hyperfine splitting $A_{\parallel}$ decreased as the perpendicular component $A_{\perp}$ increased. Both these changes in splitting reduced the order parameter as defined in Eqn. 1. The stratum corneum sample gave a similar spectrum at 20 °C (Fig. 4c), to that of the liposomal suspensions (Fig. 4a). The application of DecMSO 1% w/w brought about spectral changes similar to those described above.

Fig. 5 shows the relationship between the order parameter and the enhancer concentration for liposomal suspensions at 20 °C. Brij 36T, DecMSO and oleic acid, decreased the order parameter. This decrease was initially concentration dependent but became concentration independent on reaching a certain degree of disorder (different for each enhancer). DecMSO had the most pronounced effect on S followed by Brij 36T then oleic acid. NM2P had no effect on the order parameter. The maximum concentrations tolerated by the lipid bilayers also differed according to the enhancer used. Oleic acid was the most disruptive, solubilizing the bilayers at concentrations $> 4\%$ w/w. This solubilization was exhibited as a sudden decrease in the turbidity of the liposomal suspensions (Anzai et al., 1980) and poor forma-

tion of liposomal sediments upon centrifugation. A maximum concentration for NM2P was not determined; that for Brij 36T was approx. 9% w/w and DecMSO approx. 35% w/w.

Fig. 6 shows how S varied with temperature. At 20 °C in pure DPPC samples, S had a value of approx. 0.88 which decreased to approx. 0.58 at 55 °C. (At high temperatures the structures were therefore still quite ordered.) Samples were treated with oleic acid 2% w/w, Brij 36T 4% w/w, NM2P 20% w/w or DecMSO 28% w/w. NM2P had no effect on S and the curve (not shown) was the same as that for 'no enhancer'. The other 3 enhancers modified the shape of the curve, by decreasing S, more markedly at low temperatures than at high. At low temperatures the effect of the enhancers was the same as before: DecMSO > Brij 36T > oleic acid. At temperatures, above the T_c of the pure lipid, there was no significant difference between any of the samples.

In pure DPPC bilayers the phase transition occurs over a narrow temperature range, 1–2 °C. This sharp transition from a gel to liquid crystalline phase is due to the co-operativity between the phospholipid molecules whereby the chain 'melt-

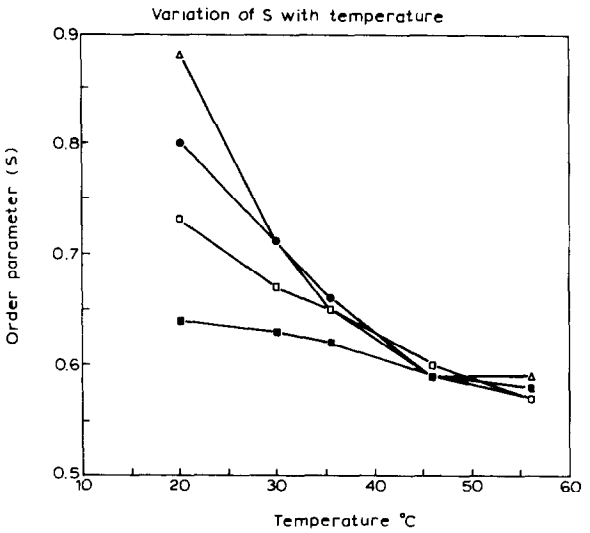


Fig. 6. Variation of S with temperature for DPPC liposomes treated with: no enhancer (△); oleic acid (●); Brij 36T (□) and DecMSO (■).

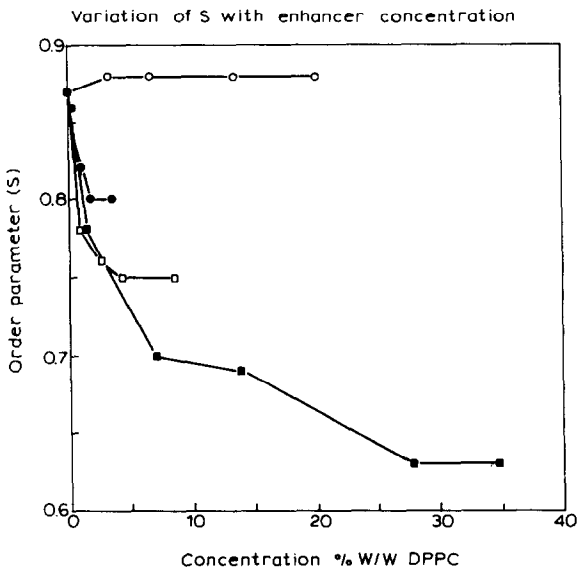


Fig. 5. Variation of S with enhancer concentration at 20 °C for DPPC liposomes treated with: NM2P (○); oleic acid (●); Brij 36T (□) and DecMSO (■).

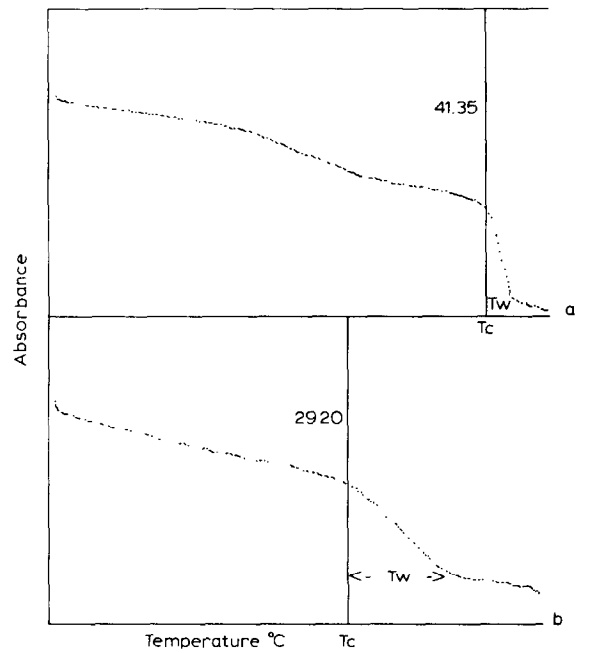


Fig. 7. Traces of absorbance vs temperature. (a) DPPC liposomes; (b) DPPC liposomes treated with an enhancer (see text).

ing' of one molecule encourages others in the nearby vicinity to undergo a similar change. Brij 36T, DecMSO and oleic acid decreased the T_c and increased the transition width (T_w), the temperature range over which the transition took place (see Fig. 7a, b). The decrease in the T_c suggests that these enhancers caused some prior 'fluidisation' of the phospholipid hydrocarbon chains and hence a lower temperature was required to complete the process and bring about a subsequent phase transition. The increase in T_w suggests weakened co-operativity within the lipid bilayer as a result of enhancer molecules intercalated between adjacent phospholipid molecules (Inoue et al., 1986). NM2P had no effect on the T_c nor the T_w.

Discussion

Membrane fluidity refers to the relative ease with which a molecule can move in a bilayer system. It can be measured in terms of the degree of disorder and/or the rates of molecular motion of the hydrocarbon chains of an incorporated spin label. An increase in fluidity is usually accompanied by a decrease in order and an increase in the rates of molecular motion. The central region of a bilayer is more fluid than the region near the polar head groups. The region probed by a nitroxide moiety depends on the position of the nitroxide group relative to the carboxyl group of the spin label. 5-DS probes the bilayer near the top of the hydrocarbon chains where the label is particularly sensitive to slight increases in its motional freedom. The order parameter S is a measure of the distribution of molecular orientations of the spin label and gives some indication of the degree of order within the bilayer.

The results of the present work suggest that certain penetration enhancers are capable of inducing membrane disorder. Of the 4 enhancers studied DecMSO brought about the greatest degree of disorder within the intact membrane. In addition, a much higher percentage of DecMSO (w/w DPPC) was tolerated by the lipid bilayer than Brij 36T and oleic acid. This difference in the maximum tolerated concentrations may be due to differences in partition coefficient. (The exact amount of enhancer remaining in the lipid bilayer upon hydration, was not determined due to difficulties in the analysis of these compounds.) Alternatively the molecular structure and conformation of the enhancers may be the more important factors. Oleic acid has a cis double-bond in the centre of its hydrocarbon chain. This produces a 'kink' in the chain which presumably has an adverse effect on the ordered packing of the phospholipid molecules. Brij 36T and DecMSO are both straight chain molecules but the smaller molecular weight of DecMSO could mean that it is more easily accommodated within the bilayer. NM2P is a cyclic molecule with no long hydrocarbon chain. The presence of a long chain capable of penetrating the hydrophobic core of a bilayer, may be a necessary attribute if a molecule is to bring about disorder.

An increase in temperature leads to a decrease in order and an increase in the rate of molecular motion. There is a change in the orientation of the phospholipid molecules with respect to a reference axis perpendicular to the bilayer surface and also a change in the relative probabilities of trans and gauche conformational isomers (Meier et al., 1982; Nagle and Scott, 1978). In the gel phase the hydrocarbon chains are relatively rigid, with an all-trans conformation and a uniform tilt. In the liquid crystalline phase the chains are flexible with a greater probability of gauche conformations and no net tilt. From our results, it appears that the potential perturbation of the membrane by an enhancer is reduced at temperatures above the T_c. At these temperatures the bilayer is already in a more fluid state and the enhancers are unable to induce any further increase in fluidity within the intact multilamellar unit. At low temperatures when the phospholipid molecules are tightly packed, the presence of enhancer molecules in the bilayer has a pronounced effect on the fluidity.

From the results it is feasible that the mode of action of Brij 36T, DecMSO and oleic acid in enhancing percutaneous absorption involves the fluidisation of intercellular lipids. NM2P probably acts via a different mechanism. The similarity between the liposomal spectra and that of the stratum corneum, suggest that DPPC multilamel-

lar liposomes are a good model of the structured intercellular lipids of the stratum corneum.

Acknowledgements

We would like to thank SERC and Boots Co. plc for a case award for C.L.G.

References

Anzai, K., Utsumi, H., Inoue, K., Nojima, S. and Kwan, T., Electron spin resonance studies on the interaction between liposomal membranes and Triton X-100. *Chem. Pharm. Bull.*, 28 (1980) 1762–1767.

Ashton, P., *Surfactant Effects in Percutaneous Absorption*, University of Wales, Ph.D. Thesis, 1987.

Bangham, A.D., Standish, M.M. and Watkins, J.C., Diffusion of univalent ions across the lamellae of swollen phospholipids. *J. Mol. Biol.*, 13 (1965) 238–252.

Barry, B.W., Mode of action of penetration enhancers in human skin. *J. Controlled Release*, 6 (1987) 85–97.

Egret-Charlier, M., Sanson, A., Ptak, M. and Bouloussa, O., Ionization of fatty acids at the lipid–water interface. *FEBS Lett.*, 89 (1978) 313–316.

Elias, P.M., Epidermal lipids, barrier function, and desquamation. *J. Invest. Dermatol.*, 80 (1983) 44s–49s.

Hubbell, W.L. and McConnell, H.M., Orientation and motion of amphiphilic spin labels in membranes. *Proc. Natl. Acad. Sci.*, 64 (1969) 20–27.

Hubbell, W.L. and McConnell, H.M., Molecular motion in spin-labeled phospholipids. *J. Amer. Chem. Soc.*, 93 (1971) 314–326.

Inoue, T., Miyakawa, K. and Shimozawa, R., Interaction of surfactants with vesicle membrane of dipalmitoylphosphatidylcholine. Effect on gel-to-liquid-crystalline phase transition of lipid bilayer. *Chem. Phys. Lipids*, 42 (1986) 261–270.

Kligman, A.M. and Christophers, E., Preparation of isolated sheets of stratum corneum. *Arch. Dermatol.*, 88 (1963) 702–705.

Meier, P., Blume, A., Ohmes, E., Neugebauer, F.A. and Kothe, G., Structure and dynamics of phospholipid membranes: an electron spin resonance study employing biradical probes. *Biochemistry*, 21 (1982) 526–534.

Nagle, J.F. and Scott, H.L., Biomembrane phase transitions. *Physics Today*, 31 (1978) 38–45.

Sanson, A., Ptak, M., Rignaud, J.L. and Gary-Bobo, C.M., An ESR study of the anchoring of spin-labeled stearic acid in lecithin multilayers. *Chem. Phys. Lipids*, 17 (1976) 435–444.

Touitou, E. and Abed, L., Effect of propylene glycol, azone and *n*-decylmethyl sulphoxide on skin permeation kinetics of 5-fluorouracil. *Int. J. Pharm.*, 27 (1985) 89–98.