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INTERACTION OF VINBLASTINE SULFATE WITH ARTIFICIAL PHOSPHOLIPID MEMBRANES

A STUDY BY DIFFERENTIAL SCANNING CALORIMETRY AND SPIN LABELING

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Abstract—The effect of the antimitotic drug vinblastine sulfate has been studied on fully hydrated dipalmitoylphosphatidylcholine (DPPC) liposomes in the temperature range 0° to 60° using differential scanning calorimetry and electron spin resonance spectroscopy with two fatty acid spin labels. In the gel phase, vinblastine interacts essentially with the DPPC polar heads and induces an important disorganization of the phospholipidic bilayer. The co-operativity of the main thermal transition is decreased. In the crystal–liquid phase, the drug penetrates inside the artificial membrane and induces the formation of domains which increased thermal stability. These effects are opposite to those observed with the drug isaxonine which is used to reduce the axonal degenerating effects due to vinblastine.

Vinblastine is a lipophilic [1, 2] antitumor agent with anti-inflammatory potency. It interferes with the metabolism of nucleic acids, proteins and lipids [3] and disrupts cell microtubules and microfilaments [4, 5]. Vinblastine-activated, membrane-associated enzymes may monitor breakdown of membrane acidic phospholipids [6]. In this respect vinblastine has common features with another lipophilic anti-inflammatory drug, sulindac [7, 8]. Vinblastine, sulindac and the antibiotic gramicidin-S affect the molar enthalpy of dipalmitoylphosphatidylcholine (DPPC)‖ main transition at all compositions [9, 10] only by a few percent. In contrast cholesterol [11, 12] abolishes this transition at molar fractions higher than 25%. This observation and the qualitatively different patterns displayed by the modulation of DPPC-bilayer hydration in the gel state by either cholesterol [12] or vinblastine [13] led two of us, using differential scanning calorimetry (DSC) [14], to the assumption that the respective locations of the two substances relative to the DPPC molecules inside the bilayers may be different at a composition $x(\text{DPPC}) = 0.9$, where $x(\text{DPPC})$ designates the molar fraction of DPPC in the mixture.

However, DSC is unable to survey the behaviour of the different parts of the phospholipid molecules, information which is given by spectroscopic methods, particularly electron spin resonance and spin labeling. This method has been used largely for studying the molecular ordering and dynamics of model membranes and the effect of numerous drugs on both artificial and natural membranes [15–22]. In this paper we present results from both DSC and spin labeling. The experiments were carried out on fully hydrated (90%, w/w water), DPPC liposomes in the absence and presence of 10% vinblastine.

MATERIALS AND METHODS

Reagents. Vinblastine sulfate was a gift from Eli-Lilly (U.S.A.). Synthetic DPPC was purchased from Calbiochem, San Diego, CA. Its purity was checked by thin-layer chromatography.

Differential scanning calorimetry experiments. The samples were prepared as described in [13] in a 5 mM phosphate buffer pH 7.4. The dry mixture lipid + drug composition was 0.300 mg DPPC + 0.037 mg vinblastine sulfate or $x(\text{DPPC}) = 0.9$. To it 3.05 mg buffer was added and weighed on a microbalance. The cup was sealed and incubated at 60° for 32 hr. A sealed reference cup containing 3.05 mg water was also prepared [12–14]. Two samples were studied. One of them was annealed at 4° for 4 days.

The DSC scanner was a Du Pont de Nemours thermoanalyser 990.910 equipped with a mechanical-cooling accessory. Scanning was performed between 5 and 60° (above the DPPC-chain melting transition) at the heating rate 2 K/min. The thermogram peak areas were evaluated using a planimeter and the heats involved were deduced. The peaks limits are designated by the onset and the peak maximum temperatures. From the measured heat of mean transition the molar enthalpy of DPPC-chain melting has been deduced ignoring the presence of vinblastine.

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‖ Abbreviations used: DPPC, dipalmitoylphosphatidylcholine; DSC, differential scanning calorimetry; 5-NS, 5-nitroxide stearate; 16-NS, 16-nitroxide stearate.

Spin labeling experiments. Multilamellar liposomes were prepared as for the DSC experiments. To 200 μl of suspension, 5 μl of a 10 mM stock solution of spin label in dimethylsulfoxide was added. The label/DPPC molar ratio was 1/1000. Samples were incubated at 50° for 30 min, to allow an uniform repartition of the label in the membrane in its crystal–liquid phase. A high local probe concentration would produce exchange-broadened ESR spectra. This was not observed on experimental spectra. Thus, we can exclude the formation of spin label micelles. A control DSC experiment was carried out on a sample of spin-labeled DPPC. The spin label does not affect the thermograms.

The spin labels were purchased from Synvar. They were *N*-oxyl-oxazolidine derivatives of stearic acid named, respectively, 5-NS when the nitroxide group is located on the 5th carbon and 16-NS when it is on the 16th carbon, the carbons being counted from the acid carboxylic group.

Electron spin resonance spectra were recorded in the temperature range 0–60° at about 1° intervals using a Varian E9 spectrometer equipped with a laboratory-built temperature controller (accuracy ± 0.2°). Since the intensity of the electron spin resonance signal may be affected by some chemical reduction of the nitroxide group, especially in the high-temperature range, the signal stability was checked by recording a series of spectra on cooling the sample from 60 to 0°. The reversibility of the results for this heating-cooling experiment was satisfactory.

The principles of interpretation of spin label spectra have been developed in numerous studies [15–20]. We shall only recall the definitions of the parameters we have measured. The outer hyperfine splittings $2A_{\parallel}$ is used as a relative measurement of the rigidity of the system. For randomly oriented samples, the magnitude of $2A_{\parallel}$ generally decreases as the result of increased motional freedom along the hydrocarbon chains. The order parameter S is dependent on the frequency and amplitude of the molecular motion. The advantage of S is that it is well-known in the liquid-crystal literature and to a good approximation it varies linearly with the position of the outermost extrema of the ESR spectrum ($2A_{\parallel}$) [23–24].

When the inner hyperfine splitting $2A_{\parallel}$ can be measured, i.e. the case when the fluidity of the system becomes sufficiently high with the 5-NS, the polarity of the spin label environment can be determined from the mean value of the splitting constant aN: 1/3 $(A_{\parallel} + 2A)$. It decreases with decreasing polarity [15].

The fluidity of lecithin liposomes labeled with the 16-NS spin probe can be characterized by the parameter t_c defined by the following expression [25].

$$t_c = 6500 \times \Delta H [(h_0/h_{-1})^{\frac{1}{2}} - 1].$$

t_c is given in nanoseconds h_0/h_{-1} is the ratio of the peak heights of the middle- and high-field lines and ΔH, the width of the central line (in Tesla). When the probe motion is roughly isotropic and $t_0 < 1$ nsec, t_c provides a good estimate of the rotational correlation time, otherwise it should be regarded as a parameter which is sensitive to changes in the probe mobility [25–27].

With both spin labels, the linewidth of the central line (ΔH) gives a qualitative information about the tumbling rate of the label: ΔH increases when this rate decreases.

RESULTS

Differential scanning calorimetry experiments

Figure 1a represents the thermograms obtained on heating the fully hydrated pure DPPC. The onset of the pretransition peak is at 32.5 ± 0.25° and the peak maximum is at about 36°. The corresponding molar enthalpy is equal to 5.8 ± 0.8 kJ/mole. The onset temperature of the main transition is 41.5 ± 0.25° and its maximum is at 42 ± 0.25°. This transition molar enthalpy is equal to 34.2 ± 0.8 kJ/mole. The main transition co-operativity number is about 150.

The thermogram on Fig. 1b is obtained for mixed vinblastine/DPPC (1/9, mole/mole) liposomes 90% (w/w) hydrated. In the presence of the drug, no pretransition is visible. The onset of the main transition peak is at 39.5 ± 0.25° and its maximum is located at 41.25 ± 0.25°. Its half-width equal to 2.5° corresponds to a co-operativity number equal to 37. A small peak ending at 45° is observed on Fig. 1b on the high-temperature side of the main peak. Its area is about 10% of the total area of the double peak. Using the total area we obtain for the molar enthalpy of DPPC main transition ΔH = 34.7 ± 0.8 kJ/mole.

Electron spin resonance experiments

5-NS spin label. This spin label probes the phospholipidic bilayer near its polar groups. The anchoring sites for the carboxylic group of the label in the bilayer polar interface are pH dependent as shown by the different $2A_{\parallel}$ and aN values displayed at 50°, respectively, at pH 2.9 and 9.9 (see Table 1a). Figure 2 shows characteristic spectra obtained at various temperatures and at pH 7.4. With pure DPPC they indicate a strongly immobilized system in the range 2–35° and a much more fluid system above 42°. The amplitude h_0 of the central line of

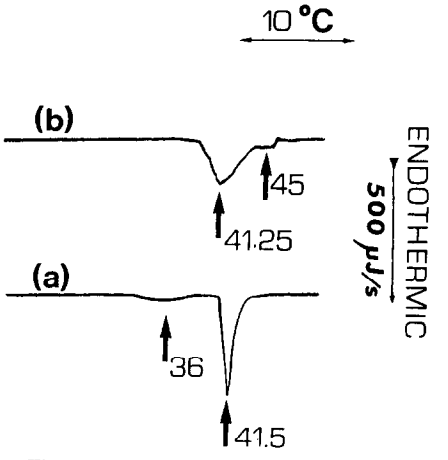

Fig. 1. Thermograms obtained by DSC on DPPC liposomes. (a) Control. (b) In the presence of 10% (mol/mol) vinblastine sulfate.

Table 1. Characteristic values of the spectral parameters measured with the 5-NS spin label. (a) Values of $2A_{\parallel}$ and aN in the crystal–liquid phase at 50°

	Control DPPC pH 2.9	Control DPPC pH 9.9	DPPC + Vinblastine pH 7.4	DPPC + Vinblastine pH 7.4
$2A_{\parallel}$ (mT)	4.075	4.80	4.76	4.08
aN (mT)	1.415	1.475	1.470	1.420

(b) Values of $2A_{\parallel}$ and S at selected temperatures

	I(°) 2		20		33		45	
	Control	Vinblastine	Control	Vinblastine	Control	Vinblastine	Control	Vinblastine
$2A_{\parallel}$ (mT)	6.50	6.26	6.26	5.78	5.96	4.20	4.76	4.12
S	0.96	0.90	0.90	0.77	0.82	0.34	0.49	0.32

$A_{\parallel}$ is the outer hyperfine coupling constant; S is the order parameter calculated from $2A_{\parallel}$; aN is the mean isotropic splitting constant.

the spectrum (Fig. 3) displays clearly the phase transition of the bilayer. In the presence of vinblastine the parameter h_0 indicates a 4° down-shift of the main transition temperature and an increase in the transition-temperature range. This can be related to the decrease in the transition co-operativity observed by DSC.

The variations of $2A_{\parallel}$ vs temperature are shown in Fig. 4. The peak meximum temperatures observed on DSC thermograms for the pretransition and the main transition are indicated by arrows. The steep sections of the curve correspond to the first-order transitions observed by DSC. The decrease in $2A_{\parallel}$ corresponds to an increase in membrane fluidity (Table 1b).

16-*NS spin label.* This label probes the DPPC bilayer near the methyl groups of the acyl chains.

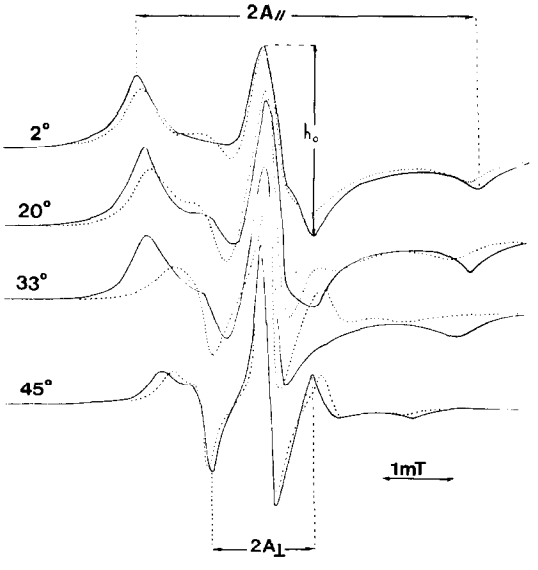

Fig. 2. Spectra obtained with the 5-NS spin label at different temperatures. Full line control DPPC. Dotted line DPPC in the presence of 10% vinblastine.

Only small spectral differences are observed with this label between the control and the vinblastine experiments. Thus Fig. 5 only shows the spectra obtained in the presence of vinblastine.

With this spin label, the amplitude of the central line h_0 is a well-defined parameter in the whole explored temperature range. Therefore it is suitable for characterizing the thermal behaviour of the system (Fig. 6). The main transition is displayed clearly at 42° by the control. No significant event is observed within the pretransition temperature range. In the presence of 10% mole/mole of vinblastine the onset of a broad transition is observed at 30° and a sharp change occurs at 45°. These transitions may correspond to the two peaks observed on the DSC thermograms (Fig. 2b).

The shift in the transition temperature is further observed on the plot of ΔH vs temperature (Fig. 7a). This parameter is inversely correlated to the tumbling rate of the label. It is observed that the fluidity is higher in the presence of vinblastine below the transition.

In the high-temperature domain it is possible to evaluate the tumbling correlation time of the label by the formula of Keith *et al.* [25]. The results are shown in the plot of log (t_c) vs $1/T$ (Fig. 7b). Taking into account the shift of the main transition temperature and the relatively low accuracy of the determination of t_c, no significant change in membrane fluidity by vinblastine is revealed at high temperature.

Table 2 indicates the characteristic spectral parameters measured with the 16-NS spin label. It should be noticed that with this label, accurate measurements can be obtained only in the temperature range 0–35° where the $2A_{\parallel}$ parameter is measurable. All these results show only a slight decrease in the ordering of the lipidic chains in the presence of vinblastine.

DISCUSSION

First we shall compare the information obtained,

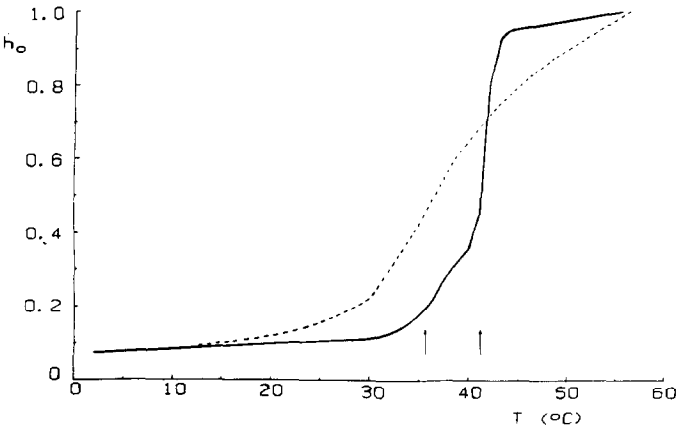

Fig. 3. Plot of the central line amplitude h_0 (see Fig. 2) measured on the 5-NS spectra as a function of temperature. The parameter h_0 is given in normalized abitrary units. Full line, control DPPC (pH 7.4). Dotted line, DPPC in the presence of 10% (mole/mole) vinblastine sulfate. The arrows correspond to the peaks observed on the DCS thermograms (see Fig. 2a).

repectively, from DSC and electron spin resonance experiments on pure DPPC liposomes. Then we shall discuss the effects observed in the presence of vinblastine at a 10% molar ratio. The results from DSC are relevant to first-order transitions in the whole-membrane system while spin labels continuously discriminate, as a function of temperature, the membrane regions where perturbations occur with a resolution of about 0.5 nm.

Behaviour of pure dipalmitoylphosphatidylcholine.

The DSC thermograms reveal a broad pretransition of low amplitude at 33° assigned to the formation of the rippled phase $P\beta'$, and to the increase in the polar head area. It is followed by the main transition at 41–42° corresponding to the gel to liquid-crystalline phase $L\alpha$ [28]. Both transitions are also observed for the control with the 5-NS spin label at pH 7.4, which probes the phospholipids near their polar parts (see Figs. 3 and 4). The decrease of $2A_{\parallel}$ at the main transition temperature corresponds to the decrease in the order parameter S.

In the liquid-crystalline phase the $2A_{\parallel}$ and aN values are pH dependent (see Table 1a). The anchoring of the spin-labeled fatty acids depends on the ionization state of the carboxylic head, as previously observed [29–30]. The protonated form (−COOH) (control at pH 2.9) penetrates the lipidic structure more profoundly than the negatively charged one ($-COO^-$) (control at pH 9.9 and pH 7.4). In the former case the $2A_{\parallel}$ parameter and the isotropic hyperfine constant aN for 5-NS are lower than in the latter case. In fact it is well-known that the closer the nitroxide groups are to the phospholipid polar heads the higher the values of the "order" parameter S. Furthermore, the isotropic hyperfine constant aN increases with the polarity of the nitroxide environment.

With 16-NS, which probes the hydrophobic central parts of the bilayers, only the main transition is revealed at 42° (Figs. 6 and 7). Therefore the gel–liquid–crystalline phase transition is simultaneously

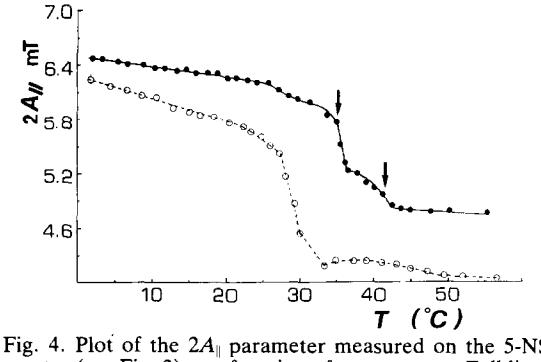

Fig. 4. Plot of the $2A_{\parallel}$ parameter measured on the 5-NS spectra (see Fig. 2) as a function of temperature. Full line, Control DPPC. Dotted line, DPPC in the presence of 10% (mole/mole) vinblastine sulfate. The arrows correspond to the peaks observed on the DSC thermograms (see Fig. 2a).

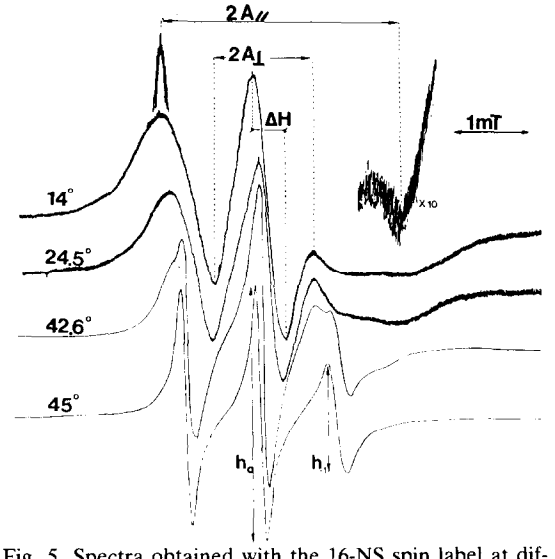

Fig. 5. Spectra obtained with the 16-NS spin label at different temperatures. (DPPC in the presence of 10% (mole/mole) vinblastine sulfate). The low- and high-field lines of the low-temperature spectra have been recorded with a 10-fold increased recorder gain in order to determine $2A_{\parallel}$ with improved accuracy.

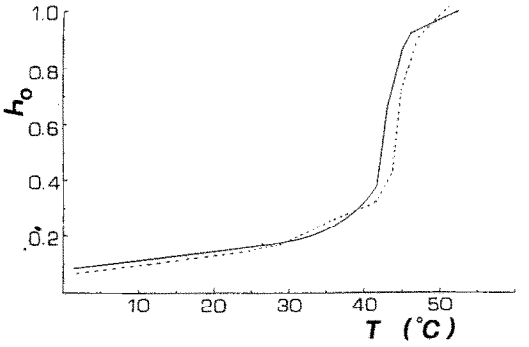

Fig. 6. Plot of the central-line amplitude h_0 (see Fig. 5) measured on the 16-NS spectra as a function of temperature. The parameter h_0 is given in normalized arbitrary units. Full line, control DPPC. Dotted line, DPPC in the presence of 10% (mole/mole) vinblastine sulfate.

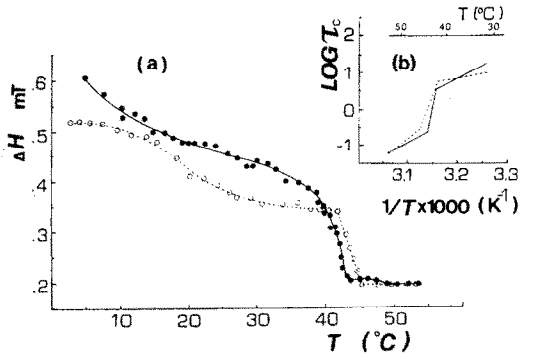

Fig. 7. (a) Plot of the peak to peak width of the central line ΔH (see Fig. 5) measured on the 16-NS spectra as a function of temperature. (b) Plot of log (t_c) vs $1/T$; the rotation correlation time t_c (in nsec) was obtained from the formula of Keith *et al.* [25]. The accuracy of this measurement is about 10%. Full lines, control DPPC. Dotted line, DPPC + vinblastine.

revealed by both spin labels. This behaviour is consistent with the large co-operativity number (150) obtained by DSC for the main transition of pure DPPC.

Effects of vinblastine

DSC reveals no first-order pretransition below 39.5° in the presence of vinblastine. Furthermore, the co-operativity of the main transition decreases about 4 times.

The 5-NS spin label shows that vinblastine affects the phospholipidic bilayer well below the main transition temperature. In the gel state (0–30°), the $2A_{\parallel}$ values are lower in the presence of the drug than in its absence. This indicates a decrease in the ordering of the DPPC acyl chains in the neighbouring of the nitroxide group.

In the low-temperature range, the acyl chain organization in the central part of the bilayer is very slightly modified, since the 16-NS spectra are practically identical in control and vinblastine-treated liposomes. Therefore below 30°, vinblastine mainly affects the DPPC polar heads.

That is further confirmed by the considerable modifications of the thermal behaviour of the liposomes in the transition-temperature range. For example, the order parameter near the polar heads (5-NS) at 33° in the presence of vinblastine is decreased to the value measured in the hydrophobic central part for the control at the same temperature. Finally, the order parameter determined with the 5-NS label in the high-temperature domain (in the liquid-crystalline state) is considerably decreased by vinblastine. Moreover, we notice that the $2A_{\parallel}$ and aN values in the presence of vinblastine (at pH 7.6) are about equal to those obtained for the control at pH 2.9 (COOH form).

These facts lead to the hypothesis that a low temperature, vinblastine perturbates the polar heads in their gel phase, probably by dipolar interactions and bilayer deformation.

At pH 7.4 vinblastine is a hydrophobic cation and spin labels are hydrophobic anions. It is well-known that the association of these species is very strong. In our system, the ratio vinblastine/spin label is 100/1. Therefore it may be that the anionic label is selectively located near the vinblastine molecules. The second-order transition observed with 5-NS at 28–32° (not revealed by DSC experiments) may be explained by a motional change of the spin label due to its deeper penetration in the hydrophobic core in relation to the structural modifications of the DPPC polar heads in the neighbouring of the vinblastine molecules.

Such an association of the drug is confirmed by the information given by DSC experiments and the 16-NS spin label. The two peaks displayed by the thermogram (Fig. 1) correspond to two types of DPPC domains one pure and one mixed with vinblastine. The E.S.R. experiments only indicate one type of label environment (no transition revealed at 42° but only at 45°). We have to admit that the label is concentrated near one of the two types of domain revealed by DSC thermograms, namely the one melting at 45° and mixed with vinblastine.

In summary, it seems interesting to compare our results with the theoretical predictions of Jähnig concerning the interactions of proteins with phospho-

Table 2. Characteristic values of the spectral parameters measured with the 16-NS spin label, as a function of temperature in the gel phase. (In the crystal–liquid phase, $2A_{\parallel}$ is longer more defined)

	T(°)							
	6		14		24		35	
T°	Control	Vinblastine	Control	Vinblastine	Control	Vinblastine	Control	Vinblastine
$2A$	4.78	4.68	4.48	4.42	4.32	4.18	4.08	4.08
S	0.53	0.52	0.48	0.47	0.43	0.42	0.37	0.37

lipid membranes. Accordingly, under the influence of proteins with increasing content, the phase transitional region appears broader and the transition temperature may be shifted upward or downward relative to the control transition temperature, while the phospholipid fluidity decreases [31–33].

Furthermore we want to notice the almost opposite effects of isaxonine and vinblastine. In fact isaxonine has been used to reduce the secondary axonal degenerating effects induced by vinblastine cancer therapy [34–38]. At low concentrations isaxonine affects mainly the lipid–water interface by inducing a modification of the ionization state of the spin label, which becomes completely ionized, and by enhancing the lipid organization [18], in contrast to vinblastine.

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REFERENCES

1. N. Neuss, *Bull. Soc. Chim. France* 1509 (1963).
2. I. S. Johnson, J. G. Armstrong, M. Gorman and J. P. Burnett, *Cancer Res.* **23**, 1390 (1963).
3. W. A. Creasey, K. G. Bensch and S. E. Malawista, *Biochem. Pharmac.* **20**, 1579 (1971).
4. R. J. Owellen, A. H. Owens and D. W. Donigian, *Biochem. biophys. Res. Commun.* **47**, 685 (1972).
5. R. J. Owellen, D. W. Donigian, C. A. Hartke and F. O. Hains, *Biochem. Pharmac.* **26**, 1213 (1977).
6. F. Schroeder, R. N. Fontaine, D. J. Feller and K. G. Weston, *Biochim. biophys. Acta* **643**, 76 (1981).
7. S. S. Fan, and T. Y. Shen, *J. med. Chem.* **24**, 1197 (1981).
8. S. B. Hwang and T. Y. Shen, *J. med. Chem.* **24**, 1202 (1981).
9. W. Pache, D. Chapman and R. Hillaby, *Biochim. biophys. Acta* **255**, 358 (1972).
10. L. Ter-Minassian-Saraga, G. Madelmont, C. Hort-Legrand and S. Métral, *Biochem. Pharmac.* **30**, 411 (1981).
11. B. D. Ladbrooke, R. M. Williams and D. Chapman, *Biochim. biophys. Acta* **150**, 333 (1968).
12. L. Ter-Minassian-Saraga and G. Madelmont, *FEBS Lett.* **137**, 137 (1982).
13. L. Ter-Minassian-Saraga and G. Madelmont, *Biochim. biophys. Acta* **728**, 394 (1983).
14. L. Ter-Minassian-Saraga and G. Madelmont, *J. Colloid Interface Sci.* **85**, 375 (1982).
15. L. J. Berliner, *Spin Labeling : Theory and Applications.* Academic Press, New York (1976).
16. D. Marsh, in *Membrane Spectroscopy* (Ed. E. Grell), pp. 50–142. Springer, Berlin (1981).
17. J. Seelig and A. Seelig, *Q. Rev. Biophys.* **13**, 19 (1980).
18. F. Berleur, V. Roman, D. Jaskierowicz, F. Leterrier, A. Esanu, P. Braquet, L. Ter-Minassian-Saraga and G. Madelmont, *Biochem. Pharmac.* **33**, 2407 (1984).
19. F. Leterrier, F. Berleur, D. Daveloose and J. Viret, *Biorheology,* Suppl. 1, 309 (1984).
20. F. Leterrier and F. Berleur *Techniques Avancées en Hémorhéologie* (Ed. *DPIC-INPL*), pp. 636–673. Nancy, France (1984).
21. D. Daveloose, M. Sablayrolles, D. Molle and F. Leterrier, *Biochem. Pharmac.* **31**, 3949 (1982).
22. W. K. Surewicz, *Biochim. biophys. Acta* **692**, 315 (1982).
23. J. Seelig, *J. Am. chem. Soc.* **92**, 3881 (1970).
24. W. Hubbell and H. M. McConnell, *J. Am. chem. Soc.* **93**, 314 (1971).
25. A. D. Keith, G. Bulfield and W. Snipes, *Biophys. J.* **10**, 618 (1970).
26. D. Kivelson, *J. chem. Phys.* **33**, 1094 (1960).
27. T. J. Stone, T. Buckman, P. L. Nordio and H. M. McConnell, *Proc. natn. Acad. Sci. U.S.A.* **54**, 1010 (1965).
28. M. J. Ruocco and G. G. Shipley, *Biochim. biophys. Acta* **691**, 309 (1982).
29. A. Sanson, M. Ptak, J. L. Rigaud and C. M. Gary-Bobo, *Chem. Phys. Lipids* **17**, 435 (1976).
30. M. Ptak, M. Eget-Charlier, A. Sanson and O. Boulousso, *Biochim. biophys. Acta* **600**, 387 (1980).
31. F. Jähnig, *Biophys. J.* **36**, 329 (1981).
32. F. Jähnig, *Biophys. J.* **36**, 347 (1981).
33. F. Jähnig, *Molec. Cryst. Liq. Cryst.* **63**, 157–170.
34. A. Huguelin, Y. Legrain and M. Bondoux-Jahan, *Experientia* **35**, 625 (1979).
35. A. Huguelin, T. Tarrade, M. Istin and R. Coelho, *C.R. hebd. Acad. Sci., Paris* **285**, 1339 (1977).
36. R. Coelho and M. Istin, *Nouv. Presse. Med.* **11**, 1227 (1982).
37. G. Duhamel and Y. Parlier, *Nouv. Press. Med.* **11**, 1254 (1982).
38. P. Guiheneuc, J. Ginet, J. Y. Grolleau and P. Rojouan, *Nouv. Press. Med.* **11**, 1257 (1982).