Biochemical Pharmacology, Vol. 33, No. 15, pp. 2407–2417, 1984.
Printed in Great Britain.

0006-2952/84 $3.00 + 0.00

ISAXONINE BASE IS A STRONG PERTURBER OF PHOSPHOLIPID BILAYER ORDER AND FLUIDITY—A DIFFERENTIAL SCANNING CALORIMETRY AND SPIN LABELING STUDY

FRANÇOIS BERLEUR,* VINCENT ROMAN,* DONALD JASKIEROWICZ,* FRANÇOIS LETERRIER,* ANDRÉ ESANU†, PIERRE BRAQUET†, LISBETH TER-MINASSIAN-SARAGA‡ and GEORGETTE MADELMONT‡

*Divisions de Biophysique et de Radiobiologie & Radioprotection du Centre de Recherches du Service de Santé des Armées, F92141 Clamart, France
†Institut Henri Beaufour, 17, avenue Descartes, F 92350 Le Plessis Robinson, France
‡Physicochimie des surfaces et des membranes, UER Biomédicale, 45 rue des Saints Pères, F 75270 Paris Cédex 06, France

(*Received* 29 *November* 1983; *accepted* 6 *February* 1984)

Abstract—The effects of the neurotropic drug isaxonine on fully hydrated dipalmitoyl-phosphatidyl-choline (DPPC) bilayers has been studied in the temperature range 0°–60°, using differential scanning calorimetry and electron spin resonance spectroscopy, with two stearic acid spin labels. At low concentration (1% mol/mol), isaxonine is trapped in the polar interface and enhances the phospholipid multibilayers organization in the gel state. In contrast, at high concentration (30% mol/mol), the drug disorganizes the phospolipidic structures and may induce domain formation by phase separation. The strong interactions of isaxonine at the lipid–water interface change the ionization state of the stearic acid spin labels which become totally ionized. Then isaxonine acts as a modifier of the surface pH of the bilayer. The strong membrane effects of isaxonine may explain in part its pharmacological properties *in vivo*.

Isaxonine, *N*-isopropyl-amino-2-pyrimidine, is a neurotropic molecule, introduced in 1977, and an artificial nervous growth factor [1, 2]. It was found that its pharmacological action involves a binding on receptor sites on tubulin [3], the major component of microtubules of the axonal cytoskeleton. It behaves as an antagonist of colchicine, vincristine and vinblastine by inducing a protective effect against the distal axonal degeneration following vincristine and vinblastine therapy [4, 5].

It seemed of interest to check if this protective effect could also involve physico-chemical changes in the neurolemma. Such changes have been shown to perturb the physiological state of numerous types of membranes [6–12]. In an attempt to explore the interaction of isaxonine with membrane bilayers, a model study with an artificial lipid membrane has been undertaken.

Two complementary methods, electron spin resonance (spin labeling) and differential scanning calorimetry, have been used in this work [13]. In fact, differential scanning calorimetry (DSC) allows study of the changes in the model phospholipid membrane thermodynamic state [14–16]. This macroscopic approach does not localize these changes on a molecular level, whereas spin labeling provides a description of the local ordering and/or 'fluidity' of the phospholipids acyl chains with a spatial discrimination (resolution) of the order of 0.5 nm [8, 9, 17–20].

In this paper, we present results carried out on fully hydrated (90% w/w water) DPPC liposome multibilayers, in the absence (control) and in the presence of 1% and 30% (mol/mol) isaxonine. This report clearly points out that isaxonine induces important changes in the molecular organization of artificial lipid membranes. In addition to serving as a model relative to hydrophobic drugs, it is hoped that isaxonine might provide a useful example for the understanding of lipid–drug interactions in membrane bilayers [10–12].

MATERIALS AND METHODS

Reagents and sample preparation. Synthetic Lα dipalmitoyl phosphatidyl-choline (DPPC) of 99% purity was purchased from Sigma. The purity was checked by thin-layer chromatography. Isaxonine base was a gift from I.H. Beaufour Laboratories. Its formula is shown in Fig. 1. It has a solubility of 6 g per 100 ml of water and its pKa is about 4.

The samples were prepared according to Bangham [21], in a 5 mM phosphate buffer pH 7.0. The dry mixture lipid + drug composition has been adjusted at the molecular ratios (Isaxonine/DPPC) 1/100 and 30/100. The multibilayer liposomes were labeled with stearic acid nitroxide derivatives on which the *N*-oxyl-oxazolidine was attached either to the C-5 or the C-16 carbon of the acyl chain, and named respectively 5NS and 16NS, the carbon being counted from the carboxylic group. Spin label probes were purchased from Syva (Palo Alto, U.S.A.).

DSC experiments. The DSC scanner was a Du Pont de Nemours thermo-analyser 990.910 equipped with a mechanical cooling accessory. Scannings were performed between 5° and 60°, at the heating rate of

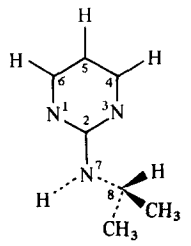

Fig. 1. Developed formula of isaxonine base.

2 K/min. The thermograms display high temperature peaks located above 20°C (Fig. 2). They correspond to the well known pretransition and gel–liquid crystal main transition. The peak characteristics are designated by their onset and maximum temperatures. The peak areas were measured using a planimeter in order to calculate the heats involved. From the measured heat of the mean transition the molar enthalpy of DPPC chain melting has been deduced ignoring the presence of isaxonine.

Spin labeling experiments. Multilamellar liposomes were prepared as for the DSC experiments. To 200 μl of the suspension, 5 μl of a 10 mM stock solution of spin label in dimethylsulfoxide was added. The label/DPPC molar ratio was 1/1000. The sample was incubated at 50° for 30 min. A control DSC experiment was carried out on a sample of spin labeled DPPC. The spin label did not affect the thermograms.

Electron spin resonance spectra were recorded in the temperature range 0°–60° at about 1° intervals using a Varian E 109 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 in cooling the sample from 60° down to 0°. The reversibility of the results for this heating–cooling experiment was satisfactory.

The principles of spin label spectra interpretation have been developed in numerous studies [17–20]. We shall recall only the definition of the parameters we have measured. The outer ($2A\|$) and inner ($2A\perp$) hyperfine splittings are used as relative measurements of the rigidity of the system. A larger value of $2A\|$ (or a smaller one of $2A\perp$) corresponds to a more rigid environment of the label. From these splitting values one obtains the order parameter S [22–27]:

$$S = \frac{A\| - A\perp}{Azz - (Axx + Ayy)/2},$$

where $Azz = 3.36$ mT, $Axx = 0.63$ mT and $Ayy = 0.58$ mT [17–19]. The isotropic hyperfine coupling constant a_n is obtained from the relation $(A\| + 2A\perp)/3$ [17–19, 27]. When $2A\perp$ cannot be determined, that is the case at low temperature with the 5NS spin label, the order parameter can be estimated from the value of $2A\|$ only [17].

The classical models describing the motion of fatty acid nitroxides in phospholipid bilayers assume that

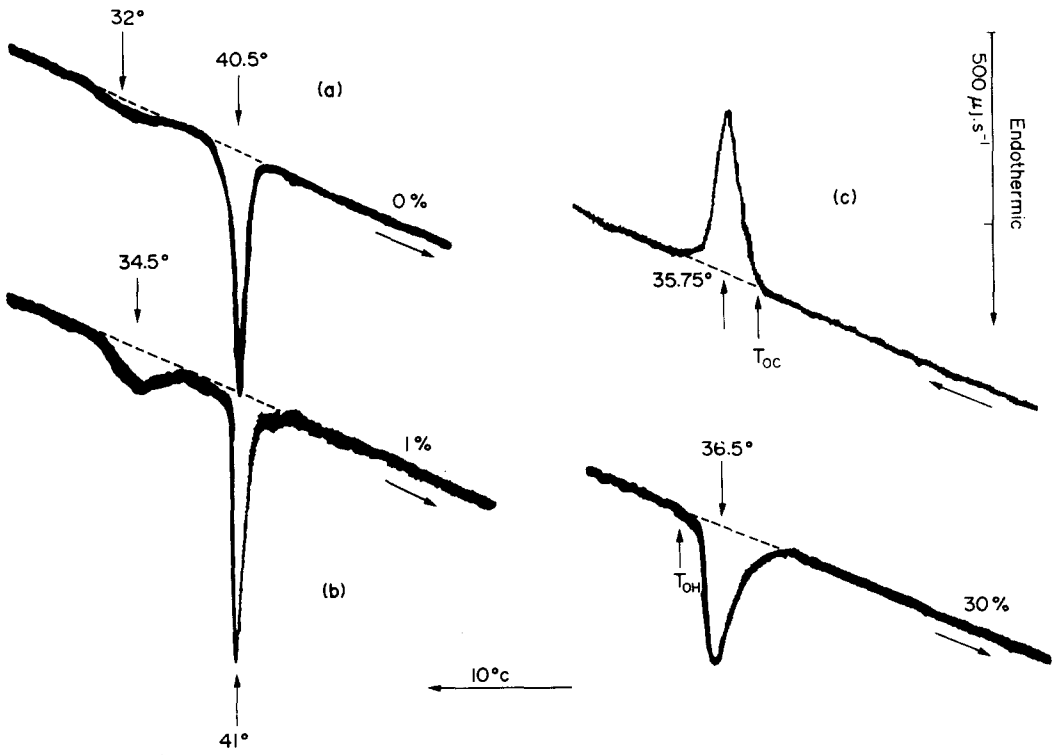

Fig. 2. DSC Thermograms of fully hydrated (90%, w/w) DPPC bilayers: (a) Control DPPC; (b) 1% isaxonine (mol/mol); (c) 30% isaxonine (mol/mol). →: heating and ←: cooling modes.

the spin labels rotate rapidly around their long molecular axis while simultaneously undergoing a restricted 'wobble' motion either in the confines of a hard cone [17, 18, 22, 28–23] or preferentially limited in a ring [13, 22, 29, 32, 33]. The size of the cone is determined by an angle β, that of the ring by the mean angle θ, both being determined from the measurement of $2A\|$. The β angle is related to the effective volume of the spin labeled fatty acid chain while $\bar{\theta}$ is relevant to the average-time structure in the immediate environment of the acyl chains. The variations of $2A\|$ and thus of θ with temperature inform on the cohesion strength in the membrane. A larger slope of the $2A\|$ or $\bar{\theta}$ versus temperature plots ($d[A\|]/dT$ or $d\bar{\theta}^2/dT$) are related to a decrease in the cohesion strength between the membrane phospholipids [13, 22, 33]. Furthermore, the parameter $\Delta\bar{\theta}^2/\Delta T.\bar{\theta}^2$ can be related to the elastic properties of the acyl chains in the vicinity of the label [22, 23].

The wobble motion is characterized by a correlation time τ_c [33–36]. The linewidth of the central line named ΔH is inversely proportional to τ_c. If τ_c is less than 1 nsec, which is the case for the fluid hydrophobic region of the membranes probed with 16NS at high temperature, it can be evaluated with a reasonable accuracy by the formula of Keith [34]:

$$\tau_c = 0.65 \cdot \Delta H\left(\sqrt{\frac{h_0}{h_{-1}}} - 1\right)$$

where τ_c is given in nsec, h_0 and h_{-1} being respectively the amplitudes of the median and high field lines of the spectrum. The activation energy of wobble motion has been determined by the Arrhenius plot of $\mathrm{Ln}(\tau_c)$ versus $1/T$ [33, 37]. The different spectral

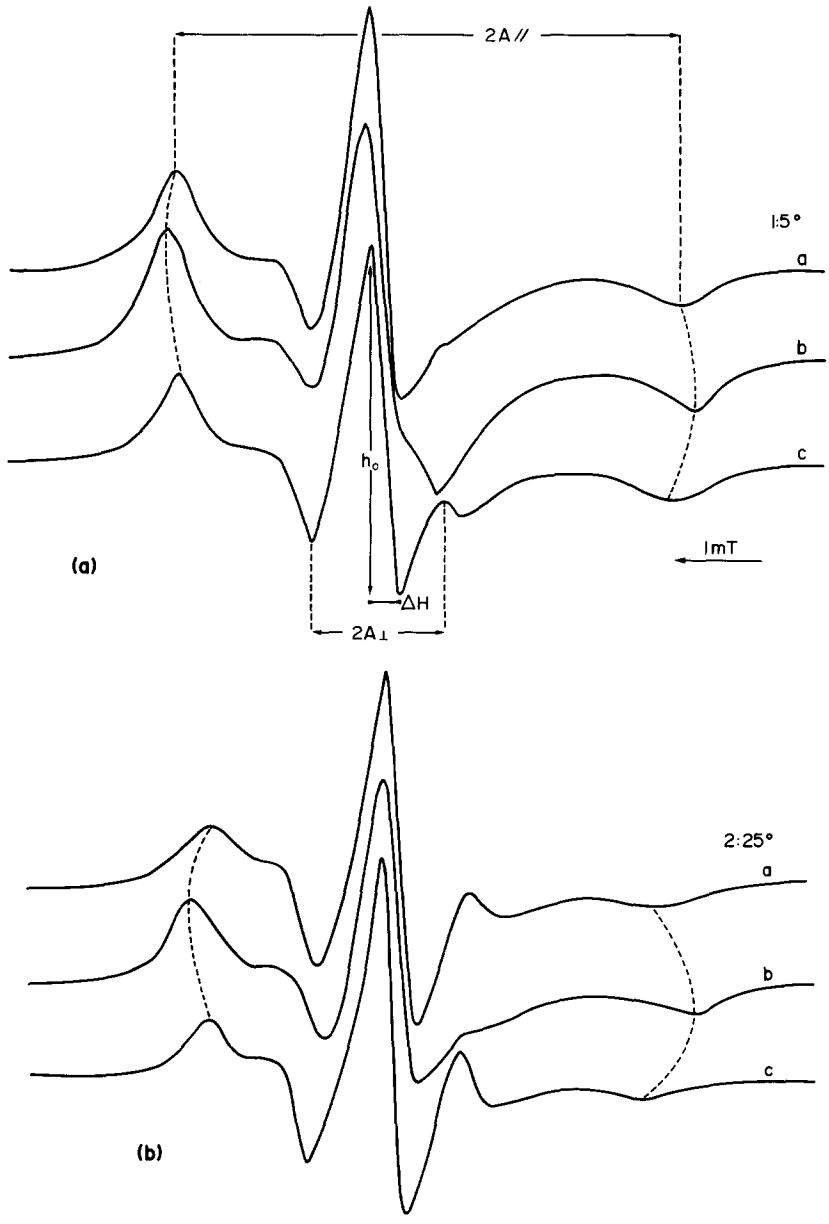

Fig. 3. *(continued)*

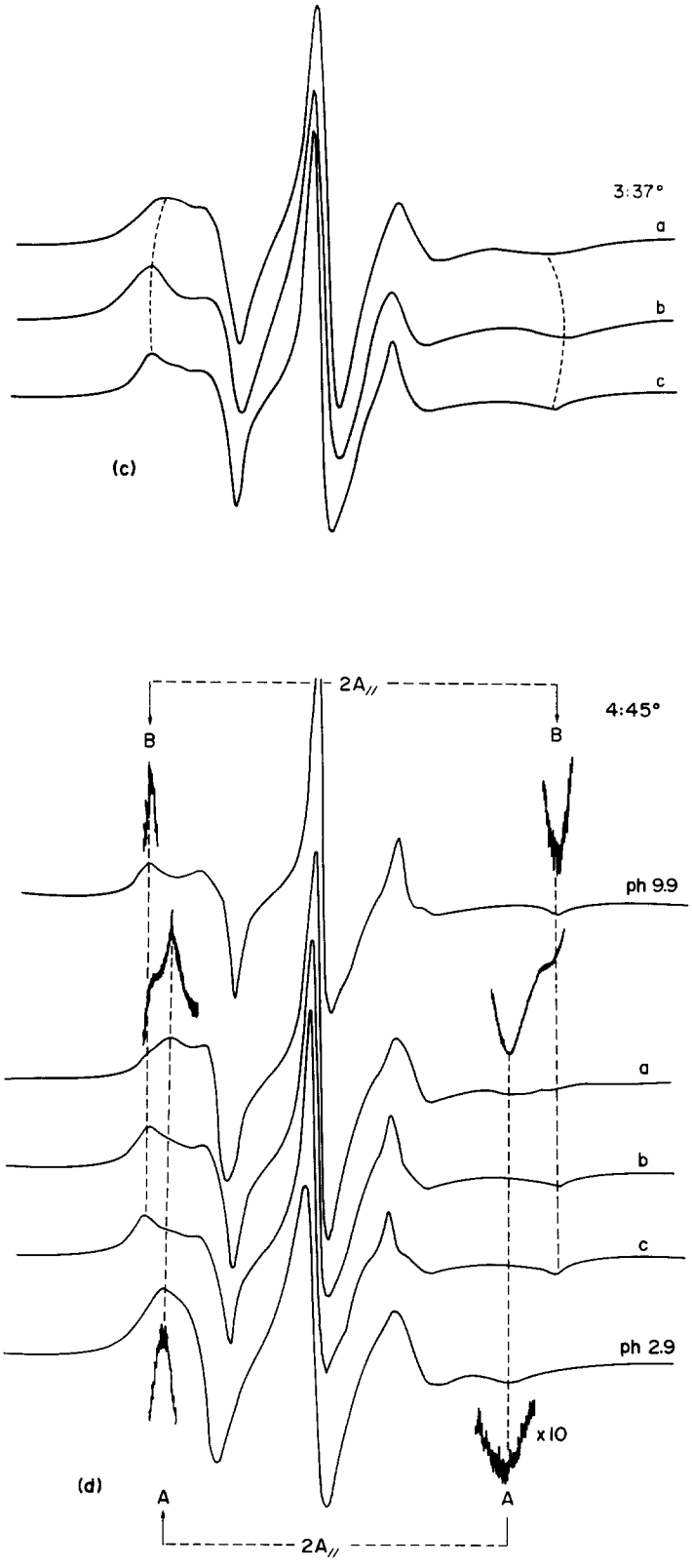

Fig. 3. Spectra obtained with the 5NS spin label at (a) 5°, (b) 25°, (c) 37° and (d) 45°. The following spectra are shown at each temperature: (A) Control DPPC pH 7.0. (B) DPPC with 1% isaxonine (mol/mol). (C) DPPC with 30% isaxonine (mol/mol). Spectra of control DPPC at pH 2.9 and 9.9 are shown at 45°.

Table 1. Temperature and cooperativity number for the gel to liquid crystal transition of DPPC isaxonine mixture given by DSC experiments

		T_p (°C)	T_s (°C)	T_l (°C)	N
Control DPPC		32.0	40.0	40.5	150
Isaxonine 1%		34.5	40.5	41.0	200
	Heating mode	/	35.0	36.50	
Isaxonine 30%					31
	Cooling mode	/	35.75	38.35	

T_p : pretransition temperature T_s: $T_{solidus}$. T_l: $T_{liquidus}$. N: cooperativity number.

parameters we have measured are defined in Figs. 3 and 7.

RESULTS

DSC experiments

Figure 2 represents the control thermograms obtained in heating the fully hydrated pure DPPC. The onset of the pretransition is at 30 ± 0.5° and its peak maximum at 32 ± 0.5°. The corresponding calculated molar enthalpy is equal to 2.6 ± 0.5 kJ/mol. The onset temperature of the main transition is 40 ± 0.25° and its maxiumum is at 40.5 ± 0.25°. The molar enthalpy of this transition is equal to 34.2 ± 0.8 KJ/mol. The cooperativity number for the main transition is about 150.

The mixed isaxonine/DPPC (1/100 mol/mol, 90% w/w hydrated) liposome thermograms reveal that in the presence of the drug, the pretransition is clearly shifted to 34.5°. The peak area is about three times that of the control. The onset of the main transition peak is weakly shifted to 40.5 ± 0.25° and its maximum located at 41 ± 0.25°. Its half-width, 30% smaller than the control, corresponds to a cooperativity number approximately equal to 200.

The thermograms for mixed isaxonine/DPPC (30/100 mol/mol, 90% w/w hydrated) liposome indicate that in the presence of the drug, only one transition occurs. Additional information has been obtained by comparing the heating and the cooling mode thermograms for the 30% isaxonine/DPPC mixture. In the heating mode, the peak onset temperature (T_{oh} = 35 ± 0.25°) is located on the solidus line and the peak maximum temperature (T_{mh} = 36.5 ± 0.25°) corresponding to the end of the DPPC transition, is located on the liquidus line (Fig. 2). We assume that the temperature range ΔT (1.5°) corresponds to a complete transition of the bilayers. In the cooling mode the peak onset (T_{oc} = 38.25 ± 0.25°) is located on the liquidus line. The peak maximum (T_{mc} = 35.75 ± 0.25°) is located on the solidus line. The temperature range ΔT (2.5°) is assigned to a complete transition of the bilayer. Such a half-width corresponds to an important decrease of the cooperativity number in the presence of 30% isaxonine (150 down to 31) (cf. Table 1). No such hysteresis phenomenon has been noticed for the control and for the 1% isaxonine samples.

Electron spin resonance experiments

5NS spin label. This spin label probes the phospholipidic bilayer near its polar groups. Figures 3(a) to 3(d) show characteristic spectra obtained at various temperatures. They indicate a strongly immobilized system in the range 2°–27° (Figs. 3(a) and 3(b)) and a much more fluid system at higher temperature (Figs. 3(c) and 3(d)). At 45° and pH 7.0 (Fig. 3(d)),

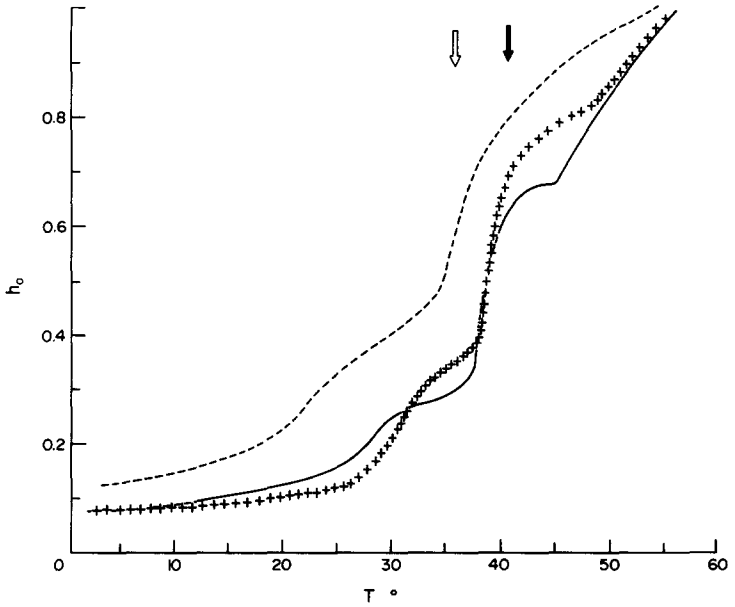

Fig. 4. Plot of the central line amplitude h_0 measured on the 5NS spectra as a function of temperature. The parameter h_0 (see Fig. 3) is given in normalized arbitrary units. —— control DPPC (pH = 7.0). +++ 1% isaxonine (mol/mol). --- 30% isaxonine (mol/mol). The main transitions observed on the DSC thermograms are indicated respectively by the full arrow for the control and 1% (mol/mol) and the open arrow for the 30% (mol/mol) isaxonine DPPC samples.

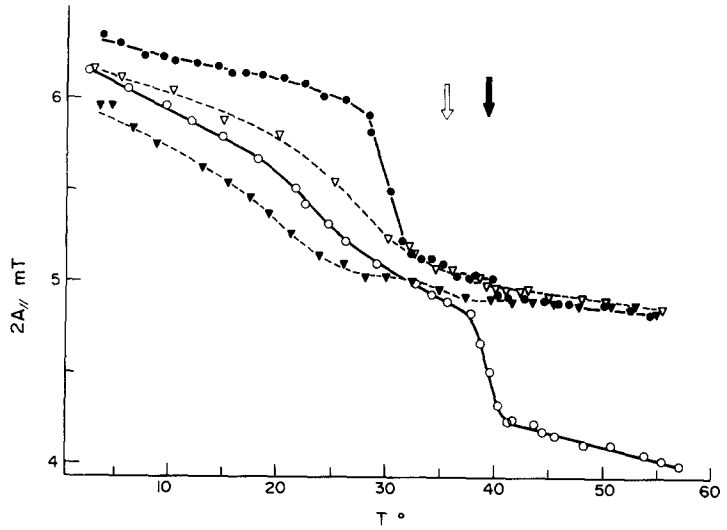

Fig. 5. Plot of the $2A\|$ parameter (see Fig. 3) measured on 5NS spectra as a function of temperature. ○——○ control pH 7.0 (DPPC). ▽- - -▽ control pH 9.9 (DPPC). ●——● 1% isaxonine (mol/mol). ▼——▼ 30% isaxonine (mol/mol). The main transitions observed by DSC are indicated by arrows as on Fig. 4.

the control DPPC spectrum displays clearly two populations of spin labels named A and B, with different values of the $2A\|$ parameter and hyperfine coupling constant a_n. These two populations indicate the existence of two well separated sites of anchoring for the carboxylic group of the label in the bilayer polar interface. This phenomenon is pH dependent, as shown by the different values displayed at 45° on the upper and lower spectra shown in Fig. 3(d), respectively at pH 2.9 and pH 9.9.

The amplitude h_0 of the central line of the spectrum (Fig. 4) is a well defined parameter in the whole explored temperature range. It displays clearly the phase transition temperature of the bilayer. At the temperature of the main transition, the increase in the slope of the parameter h_0 may be correlated to the cooperativity number measured by DSC. With 1% isaxonine, the rate of change in fluidity with temperature is smaller than the control below 25°. The pretransition is enhanced and upshifted by 2°. The slope of the main transition is larger than that of the control. In contrast, the 30% isaxonine curve is smoothed and globally shifted to lower temperature by about 4°.

Figure 5 represents the variations of the $2A\|$ parameter in various conditions. Curves with open sym-

Table 2. Characteristic values of the spectral parameters measured with 5 NS at various temperatures. $2A\|$ is the outer hyperfine coupling constant, S the order parameter calculated from $2A\|$, β the wobble angle, $\bar{\theta}$ the mean 'ring' angle, a_n the mean isotropic hyperfine coupling constant.

			3.5°	25°	35°	45°
Control	pH = 7.5	$2A\|$ (mT)	6.090	5.450	4.880	4.125
		S	0.828	0.654	0.499	0.293
		$\bar{\theta}$	19.77	28.70	35.32	43.35
		β	28.35	41.60	51.90	65.60
		a_n				1.410 ± 0.005
	pH = 9.9	$2A\|$ (mT)	6.130	5.530	5.080	4.925
		S	0.840	0.677	0.544	0.511
		$\bar{\theta}$	19.80	27.66	33.05	34.80
		β	27.30	39.98	48.3	51.10
		a_n				1.470 ± 0.005
Isaxonine	1%	$2A\|$ (mT)	6.360	6.000	5.080	4.875
		S	0.902	0.805	0.554	0.497
		$\bar{\theta}$	14.80	21.16	33.05	35.35
		β	21.04	30.29	48.30	51.98
		a_n				1.455 ± 0.005
	30%	$2A\|$ (mT)	5.980	5.130	4.950	4.890
		S	0.799	0.566	0.519	0.501
		$\bar{\theta}$	21.46	32.52	35.50	35.21
		β	30.75	47.47	50.60	51.75
		a_n				1.460 ± 0.005

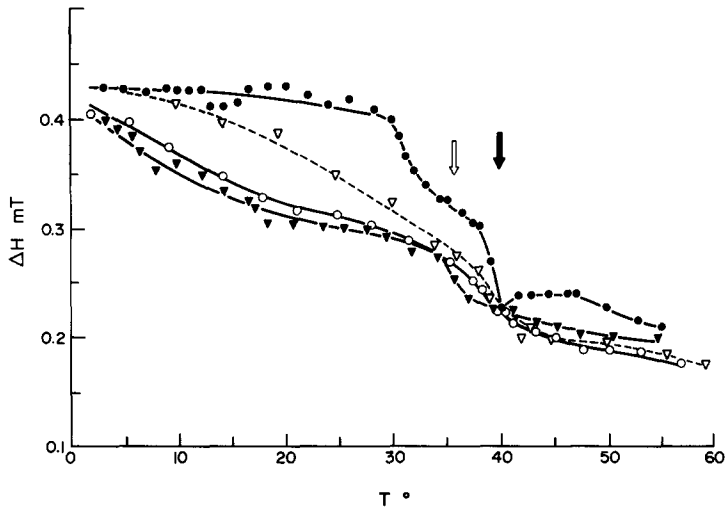

Fig. 6. Plot of the width of the central line ΔH (see Fig. 3) measured on the 5NS spectra, as a function of temperature. O——O control pH 7.0 (DPPC). ▽- - -▽ control pH 9.9 (DPPC). ●——● 1% isaxonine (mol/mol). ▼——▼ 30% isaxonine (mol/mol). The main transitions observed by DSC are indicated by arrows as on Fig. 4.

bols correspond to control DPPC respectively at pH 9.9 (open triangles) and pH 7.0 (open circles). In the liquid-crystalline phase, the $2A_{\parallel}$ values remain relatively high at pH 9.9 and corresponds to population B in Fig. 3(d). With DPPC at pH 7.0 we have reproduced the $2A_{\parallel}$ parameter variation of the predominant populations named A (cf. Fig. 3(d)). A strong decrease of this parameter is observed above the main transition temperature. The separation of the two populations A and B increases with temperature. Full symbols in Fig. 5 correspond to the effect of isaxonine at low (1% mol/mol: full circles, and high 30% mol/mol: full triangles) concentration. The opposite effect of the low and high isaxonine concentrations is more dramatic in the gel phase below 32°. With 1% isaxonine, $2A_{\parallel}$ is always higher than for the pH 7.0 control. It decreases less rapidly in the low temperature range 0–27°. It is interesting to notice a sharp transition in the range 27–33° which could be correlated to the enhancement in the pretransition peak observed on the DSC thermogram. A smooth variation between 33 and 40° is observed, followed by a weak step at 41° (temperature of the main transition), nearly five times smaller than the control one. Moreover, we notice that above 32°, the $2A_{\parallel}$ values for 1% isaxonine behave like the pH 9.9 DPPC control. In the gel phase, the $2A_{\parallel}$ parameter remains smaller in the presence of 30% isaxonine than for the pH 7.0 control DPPC. After a very smooth variation at about 35°, the curve is similar to that of the 1% isaxonine and the pH 9.9 DPPC control. The order parameter and the wobble angle values obtained from these data are indicated in Table 2.

The results for ΔH in Fig. 6 are consistent with those in Fig. 5. With 1% isaxonine (full circles) at the temperature of the main transition we notice an important decrease of ΔH with a minimum at 40°, followed by a weak but significant increase at higher temperatures. This value of ΔH at 40° is identical for both DPPC controls (pH 7.0 and pH 9.9) and for DPPC in the presence of isaxonine (either 1% or 30% mol/mol). The 30% isaxonine curve displays an interesting step at 35° and is located between 1% isaxonine and both pH 7.0 and pH 9.9 control curves in the liquid phase temperature range. It is interesting to notice that even for the ΔH parameter,

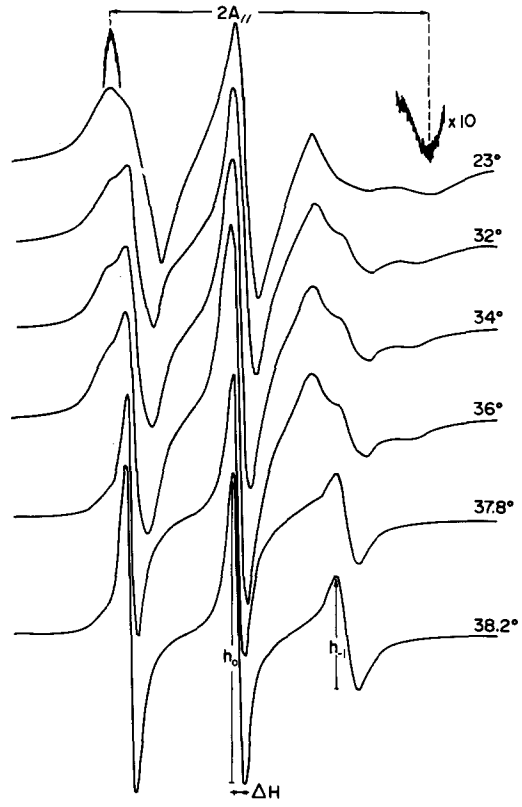

Fig. 7. Spectra obtained with the 16NS spin label at different temperatures with 30% isaxonine (mol/mol).

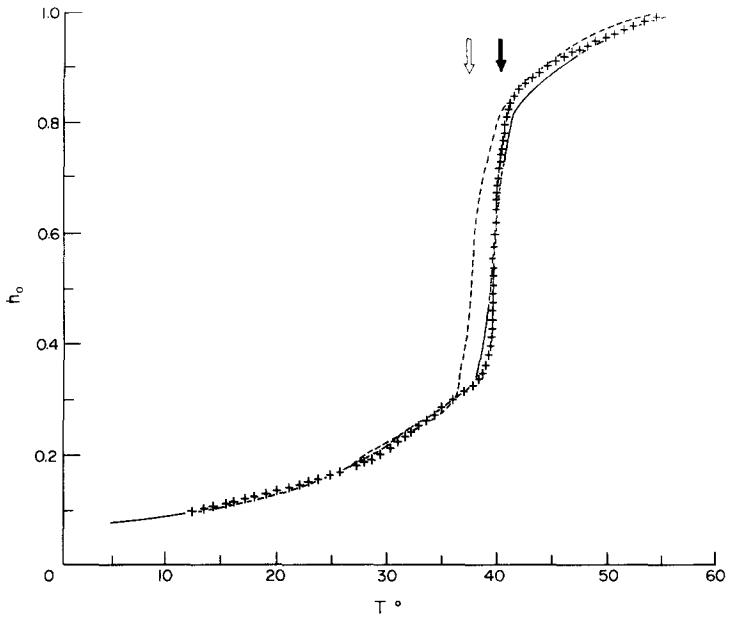

Fig. 8. Plot of the central line h_0 measured on the 16NS spectra as a function of temperature. The h_0 (see Fig. 7) parameter values are given in normalized arbitrary units. —— Control DPPC. +++ 1% isaxonine (mol/mol). --- 30% isaxonine (mol/mol). The main transitions observed by DSC are indicated by arrows as on Fig. 4.

the values corresponding to the pH 7.0 and pH 9.9 spin label populations, remain different in the gel phase.

16NS *spin label.* This spin label probes the DPPC bilayer near the methyl group of the acyl chains. Variations of the spectral shape versus temperature are shown in Fig. 7 for 30% isaxonine.

In Fig. 8, the amplitude of the central line h_0 shows that the main transition is clearly displayed at 40° for the control. No significant change in this transitition temperature is observed with 1% isaxonine. Nevertheless, an increase of the slope of the curve in the phase transition range agrees with the increase in the cooperativity number described above. The 30%

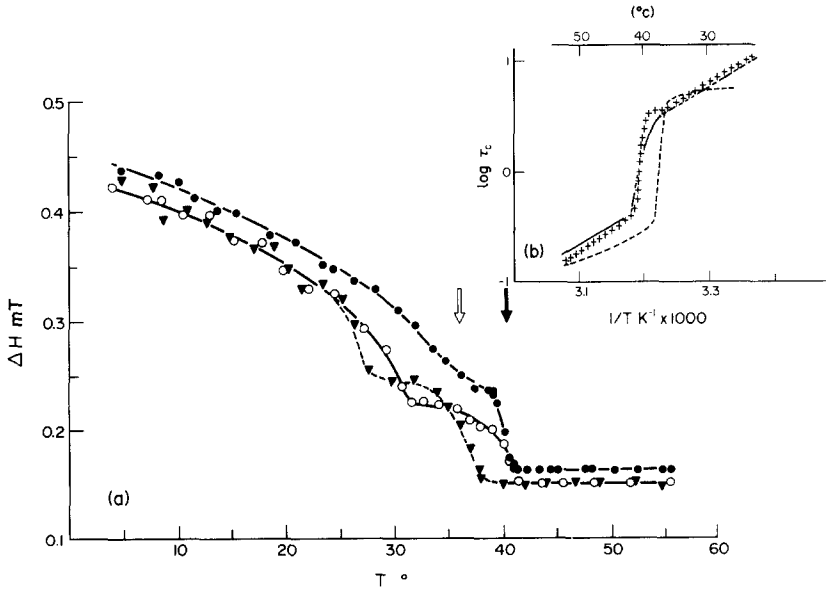

Fig. 9. (a) Plot of the width of the central line H measured on the 16NS spectra as a function of temperature (see Fig. 7). O——O Control DPPC. ●——● 1% isaxonine (mol/mol). ▼- - -▼ 30% isaxonine (mol/mol). The main transitions observed by DSC are indicated by arrows as on Fig. 4. (b) Plot of Log (τ_c) versus $1/T$. The motional correlation time in nanoseconds is obtained from the formula of Keith [34]. The accurary of this measurement is about 5%. Same symbols as on Fig. 8.

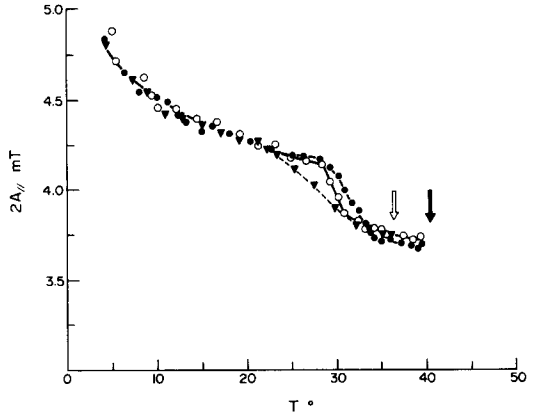

Fig. 10. Plot of the $2A\|$ parameter measured on the 16NS spectra (see Fig. 7) as a function of temperature. The main transitions observed by DSC are indicated by arrows as on Fig. 4. Same symbols as on Fig. 9a.

isaxonine curve displays a 2° shift down to lower temperatures. We notice no difference between the slope of the 30% isaxonine and the control (pH 7.0) curves. No significant event is observed within the pretransition temperature range.

These effects in the transition temperature range are further observed on the plot of ΔH versus T (Fig. 9a). This parameter is correlated to the tumbling rate of the label. Except for the end point of the transition at 41°, the ΔH values for 1% isaxonine are clearly above the control. Moreover, the phase transition is sharp and amplified. Below 25° and above 41°, the control and 30% isaxonine curves are comparable. The main transition in the presence of 30% isaxonine is shifted down to 37°. All these results can be summed up in the plot of the tumbling rate (Log τ_c) versus $1/T$ (Fig. 9b). No significant difference is noticed between 1% and the control except an increase in the slope at the transition temperature. In contrast the 30% isaxonine slope of the curve in the liquid phase is 17% lower.

An accurate measurement of the $2A\|$ parameter is only possible in the gel phase up to the onset of the main transition. Figure 10 indicates the step corresponding to the pretransition. This step is shifted up and amplified in DPPC bilayers with 1% isaxonine and smoothed between 25° and 35° in the presence of 30% isaxonine. The $2A\|$ values are identical below 25° for all concentrations (cf. Table 3).

Table 3. Characteristic values of the spectral parameters measured with 16NS for three temperatures (see Table 2)

		10°	27°	37°
Control	$2A\|\|$ (mT)	4.475	4.175	3.750
	S	0.482	0.394	0.287
Isaxonine 1%	$2A\|\|$ (mT)	4.50	4.175	3.760
	S	0.482	0.408	0.275
Isaxonine 30%	$2A\|\|$ (mT)	4.40	4.025	3.750
	S	0.467	0.360	0.290

DISCUSSION

Behaviour of pure DPPC

The DSC thermograms [13] reveal a broad pretransition of low amplitude at 32° 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 40–42° corresponding to the gel to liquid crystalline phase $L\alpha$[38]. Both transition are also observed for the control with the 5NS spin label which probes the phospholipids near their polar parts (Fig. 4). The decrease of $2A\|$ at the main transition temperature (Fig. 5) corresponds to the decrease in the order parameter S and the increase of the characteristic wobble angles (Table 2) of the acyl chains close to the polar heads. With 16NS which probes the hydrophobic central parts of the bilayers the decrease at 32° of the $2A\|$ parameter (Fig. 10) corresponds to the pretransition. The main transition is revealed at 42° (Figs. 8 and 9). Therefore the gel–liquid crystalline phase transition is simultaneously revealed by both spin labels. This behaviour is consistent with the large cooperativity number (150) obtained by DSC for the main transition of pure DPPC.

Effects of isaxonine at low concentration

The main effect induced by isaxonine at low concentration (1%) is to reinforce the structural organization of the membrane. This is indicated by the increases in the pretransition amplitude and in the main transition cooperativity as shown by DSC. The spin label results agree with those of DSC. They show the same increases in the pretransition amplitude (h_0 parameter with 5NS and $2A\|$ with 16NS) and in the slope of the spectroscopic parameters variation at the main transition. A detailed analysis of the results obtained with both spin labels allows us to define more accurately the mode of interaction of isaxonine with the different regions of the DPPC model membrane in its different thermal states (gel and liquid crystal). The predominant fact which can be deduced from this analysis is the strong increase in the rigidity of the DPPC bilayer near its polar heads in the gel state. In fact, an increase of 23% of the $2A\|$ parameter measured with 5NS is observed at 25° (see Table 2). This increase is not found in the hydrophobic core since the $2A\|$ measured with 16NS is not modified. With this spin label, only ΔH is slightly increased, indicating a decrease in the tumbling rate of the label (Fig. 9). The increase in the pretransition amplitude and temperature and the stronger modification of the spectroscopic parameters measured with 5NS than with 16NS suggest a preferential incorporation of isaxonine at the water–lipid interface.

The last hypothesis is reinforced by the modification of proportions in the two populations A and B observed with the 5NS spin label in the liquid crystalline state (Fig. 3). The anchoring of the spin-labeled fatty acids depends on the ionization state of the carboxylic head as shown by Sanson and Ptak *et al.* [39, 40] and by ourselves [13]. The protonated form (—COOH) (cf. Fig. 3(d): control at pH 2.9) penetrates in the lipidic structure more profoundly than the negatively charged one ($-COO^-$) (Fig. 3(d)

: pH 9.9). In the former case the $2A\|$ parameter for 5NS is lower and the isotropic hyperfine constant is higher than in the latter case. In fact it is well known that the nearer are the nitroxide groups to the phospholipid polar heads, the higher are the values of the order parameter. Furthermore, the isotropic hyperfine constant a increases with the polarity of the nitroxide environment [41]. At pH 7.0 in the control the spin label is predominantly present in the COOH form (Fig. 3(d)). In the presence of isaxonine, the $2A\|$ parameter for 5NS is identical to that of the control at pH 9.9 (the COO^- form) and the isotropic hyperfine splitting value (1.455 ± 0.005 mT) has an intermediate value between those of the COO^- and COOH forms. This indicates that the drug has displaced the equilibrium between both forms of the label in favour of the ionized state. This could be due to an increase in the local pH when the drug is present near the DPPC polar heads. This electrostatic interaction between the drug and the spin label could explain the increase in the order parameter also observed in the gel state of the bilayer. However, the results shown in Figs 5 and 6 and Table 2 indicate clearly that this increase is significantly larger in the presence of the drug than the one that is observed by the displacement of the label ionization equilibrium when the bulk pH is increased. Thus the label shows the perturbation induced by the drug in the whole phospholipid organization near their polar heads.

The increase in the $2A\|$ (5NS) parameter corresponds to a decrease of the cone angle β which is itself related to the mean surface occupied by the phospholipid molecules in the vicinity of the spin label [42–45]. Such a decrease in this molecular area could be due to a parallel lowering in the hydration degree of the bilayer polar part [46, 47]. This hypothesis is corroborated by the DSC results. In fact it has been shown that a decrease in the hydration level leads to the enhancement of the pretransition enthalpy, its shift to higher temperature as well as an increase in the main transition temperature [46–49], all facts which are observed in the presence of 1% isaxonine.

This lowering of the DPPC bilayer hydration can be correlated with an increase in the cohesion strength between the acyl chains. This is indicated by the weak variation in the $2A\|$ or ΔH parameters as a function of temperature in the 0–25° range. In fact the values of the $d(A\|)/dT$ or $d\bar{\theta}^2/dT$ slopes are reduced to 55% of the control ones. Concerning the elastic properties of the acyl chains, no significant change occurs in the presence of the drug since the parameter $\Delta\bar{\theta}^2/\Delta T.\bar{\theta}^2$ is practically identical to that of the control in this temperature range.

Effects of isaxonine at high concentration

At high isaxonine concentration (30% mol/mol) an important disorganization of the membrane structure is demonstrated by the down-shift of the transition temperature, its strong broadening and the correlated decrease in cooperativity (150 to 31) [50–52]. These facts concerning the transition are observed on the plots of the esr spectroscopic parameters versus temperature. It must be noticed that the transition temperature shift has not the same value for both spin labels. The shift observed with h_o or ΔH measured with 5NS has the same value (4°) as for DSC whereas it is of only 2° with 16NS. This confirms the strongest interaction of the drug with the bilayer polar part and may explain the decrease in cooperativity observed in DSC: the 'fusion' phenomenon does not occur simultaneously for all the length of the acyl chains as shown by other authors [43, 53]. It may be relevant to the hysteresis observed in DSC.

In contrast to 1% isaxonine samples, the 30% concentration induces a decrease in $2A\|$ (Fig. 5) and ΔH (Fig. 6) measured with 5NS, showing a lowering of the chain order (see Table 2) compared to the control values. Furthermore the parameter $\Delta\bar{\theta}^2/\bar{\theta}^2.\Delta T$ is divided by 1.8. Thus the elasticity of the acyl chains is decreased at 30% isaxonine in the gel state.

At 41°, in the fluid phase, the $2A\|$ parameter measured with 5NS in the presence of 30% isaxonine at pH 7.0 has the same value as that measured on the pH 9.9 control sample: this corresponds to the previously indicated displacement of the $COOH \rightleftharpoons COO^-$ equilibrium induced by the drug. The membrane disorganization is also observable in the hydrophobic region within the liquid phase, since the apparent correlation time τ_c and the tumbling activation energy are diminished respectively by about 20% and 17% at 45° [37].

In conclusion, this report demonstrates clearly the interest of the association of DSC and spin label methods for the study of the mode of interaction of a drug with a model membrane. DSC gives global information on the first order transitions and cannot be suspected to induce some artefacts since it does not use any marker. ESR affords more accurate information since the labels 'see' the local modifications of the membrane organization. However, we must be careful in the interpretation of the spectroscopic parameter variations, particularly with fatty acid spin labels. The drug may be able to induce a change in the label ionization state and thus modify their anchoring in the membrane. However, this indicates a strong interaction of the drug with the membrane–water interface.

Concerning the isaxonine effects on DPPC bilayers, all our results demonstrate a strong interaction of this drug with the polar heads. To our knowledge, it is one of the first times that a drug is shown to enhance the phospholipidic structural organization of a model membrane at such a low drug/phospholipid ratio. It seems that only the small polypeptide melittin induces analogous effects [53]. Numerous studies of drug–membrane interaction have been performed by various biophysical methods including DSC, ESR, NMR, fluorimetry and Raman spectroscopy. In the majority of cases, the studied drugs provoke some disorganization of the phospholipidic structure and moreover the effects are observed at high drug/phospholipid ratios.

It is difficult to extrapolate the results obtained on DPPC bilayers to natural membranes. It seems, however, interesting to point out that vinblastine induces opposite effects on the DPPC bilayer structure [13] (Berleur *et al.*, in preparation) and to recall that isaxonine is used in human therapeutics to treat

the secondary effects due to vinblastine in the nervous system [4, 5]. Moreover, the increase in membrane organization induced by isaxonine may change the activity of membrane-bound enzymes [6, 7].

Acknowledgements—We acknowledge the Direction des Recherches et Etudes Techniques and grants from Institut Henri Beaufour and Fondation pour la Recherche Médicale.

REFERENCES

1. A. Huguelin, Y. Legrain and M. Bondous-Jaman, *Experientia* **35**, 625 (1979).
2. A. Huguelin, T. Tarrade, M. Istin and R. Coelho, *C. R. Acad. Sci. Paris* série D, **285**, 1339 (1977).
3. R. Coelho et M. Istin, *Nouv. Presse Med.* **11**, 1227 (1982).
4. P. Guiheneuc, J. Ginet, J. Y. Grolleau and J. Rojouan, *Nouv. Press. Med.* **11**, 1257 (1982).
5. G. Duhamel and Y. Parlier, *Nouv Press. Med.* **11**, 1254 (1982).
6. F. Giraud, M. Claret, R, Bruckdorfer and B. Chailley, *Biochim. biophys. Acta* **647**, 249 (1981).
7. R. Salesse, J. Garnier, F. Leterrier, D. Daveloose and J. Viret, *Biochemistry* **21**, 1581 (1982).
8. F. Leterrier, *Act. biochim. pharmac.* **2**, 91 (1982).
9. D. Daveloose, M. Sablayrolles, D. Molle, and F. Leterrier, *Biochem. Pharmac.* **31**, 3949 (1982).
10. F. Jahnig, *J. chem. Phys.* **70**, 3279 (1979).
11. F. Jahnig, *Biophys. J.* **36**, 329, 347 (1981).
12. F. Jahnig, *Mol. Cryst. Liq. Cryst.* **63**, 157 (1981).
13. F. Berleur, Doctoral Thesis. Université Pierre et Marie Curie, Paris, 19/12/1983.
14. L. Ter-Minassian-Saraga and G. Madelmont *FEBS Lett.* **137**, 137 (1982).
15. B. Labbrooke and D. Chapman, *Chem. Phys. Lipids* **3**, 304 (1969).
16. P. D. Garn, *Thermoanalytic Methods of Investigations.* Academic Press, New York (1965).
17. L. J. Berliner, *Spin Labeling: Theory and Applications.* Academic Press, New York (1976).
18. D. Marsh, in *Membrane Spectroscopy* (Ed. E. Grell), pp. 50–142. Springer, Berlin (1981).
19. M. Ptak, *Biochimie* **57**, 483 (1975).
20. J. Seelig, *J. Am. chem. Soc.* **92**, 3881 (1970).
21. A. D. Bangham, M. M. Standish and J. C. Wattkins, *J. molec. Biol.* **13**, 238 (1965).
22. F. Leterrier, F. Berleur, D. Daveloose, and J. Viret, *Biorheology*, in press.
23. W. L. Hubbell and H. M. McConnell, *J. Am chem. Soc.* **93**, 314 (1971).
24. A. D. Keith, M. Sharnoff and G. E. Cohn, *Biochim. biophys. Acta* **300**, 379 (1973).
25. D. W. R. Gruen, *Chem. Phys. Lipids* **30**, 105 (1982).
26. J. Belle and P. Bothorel, *Biochem. biophys. Res. Commun.* **58**, 443 (1974).
27. P. Jost, L. J. Libertini, V. C. Hebert and O. H. Griffith, *J. molec. Biol.* **59**, 77 (1971).
28. S. P. Van, G. B. Birrell and O. H. Griffith, *J. Magn. Res.* **15**, 444 (1974).
29. B. J. Gaffney and H. M. McConnell, *J. Magn. Res.* **16**, 1 (1974).
30. J. Israelachvili, J. Sjosten, L. E. G. Eriksson, M. Ehrstrom, A. Graslund and A. Ehrenberg, *Biochim. biophys. Acta* **382**, 125 (1975).
31. H. Blum, J. C. Salerno and J. S. Leigh, Jr., *J. Magn. Res.* **30**, 385 (1978).
32. Y. Shimoyama, L. E. G. Eriksson and A. Ehrenberg, *Biochim. biophys. Acta* **508**, 213 (1978).
33. F. Leterrier and F. Berleur, *La Résonance paramagnétique électronique. Cours d'Hémorhéologie avancée* (Ed. J-F. Stoltz). Nancy, in press.
34. A. D. Keith, G. Bulfield and W. Snipes. *Biophys. J.* **10**, 618 (1970).
35. D. Kivelson, *J. chem. Phys.* **33**, 1094 (1960).
36. T. J. Stone, T. Buckman, P. L. Nordio and H. M. McConnell, *Proc. natn. Acad Sc. U.S.A.* **54**, 1010 (1965).
37. M. Shinitzky and M. Inbar, *Biochim. biophys. Acta* **433**, 133 (1976).
38. M. J. Ruocco and G. G. Shipley, *Biochim. biophys. Acta* **691**, 309 (1982).
39. A. Sanson, M. Ptak, J. L. Rigaud and C. M. Gary-Bobo, *Chem. Phys. Lipids* **17**, 435 (1976).
40. M. Ptak, M. Egret-Charlier, A. Sanson and O. Bouloussa, *Biochim. biophys. Acta* **600**, 387 (1980).
41. O. H. Griffith, P. J. Dehlinger and S. P. Van, *J. Memb. Biol.* **15**, 159 (1974).
42. A. G. Lee, *Biochemistry* **16**, 835 (1977).
43. A. G. Lee, *Biochim. biophys. Acta* **472**, 237 (1977).
44. J. L. Rigaud, C. M. Gary-Bobo, A. Sanson and M. Ptak, *Chem. Phys. Lipids* **18**, 23 (1977).
45. J. L. Rigaud, Y. Lange, C. M. Gary-Bobo, A. Sanson and M. Ptak, *Biochem biophys. Res. Commun.* **50**, 61 (1972).
46. L. Ter-Minassian-Saraga and G. Madelmont, *J. Colloid Interface Sci.* **81**, 369 (1981).
47. D. Chapman, in *Biological Membranes* (Eds. D. Chapman and D. F. H. Wallach), Vol. 2, pp. 91–145. Academic Press, New York (1973).
48. D. Chapman, *Adv. Liquid Crystals* **5**, 1 (1982).
49. D. Chapman, R. M. WIlliams and B. D. Ladbrooke, *Chem. Phys. Lipids* **1**, 445 (1967).
50. Bruggeman and D. L. Melchior, *J. biol. Chem.* **258**, 8298 (1983).
51. L. Ter-Minassian-Saraga and G. Madelmont, *Biochim. biophys. Acta* **728**, 394 (1983).
52. L. Ter-Minassian-Saraga and G. Madelmont, *J. Colloid. Interface Sci.* **85**, 375 (1982).
53. S. P. Verma and D. F. H. Wallach, *Biochim. biophys. Acta* **426**, 616 (1976).