Biochimica et Biophysica Acta, 555 (1979) 388–408

BBA 78460

IS AN AVERAGE VISCOSITY TENABLE IN LIPID BILAYERS AND MEMBRANES?

A COMPARISON OF SEMI-EMPIRICAL EQUIVALENT VISCOSITIES GIVEN BY UNBOUND PROBES: A NITROXIDE AND A FLUOROPHORE

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(Received December 6th, 1978)

Key words: Microviscosity; Fluorescence polarization; EPR; Nitroxide; (Lipid bilayer)

Summary

Relative variations of fluidity in bilayers and membranes are currently evaluated by numerous physical methods, but comparisons between different systems remain difficult because the effects of order (anisotropy) and fluidity are involved in the diffusion coefficients (or correlation times, or frictional coefficients) given by experiment.

The present report represents an attempt to generalize the use of isotropic liquids as viscosity standards for disordered lipidic systems. It advances a simple check to verify the quasi-isotropic behaviour of probe environments and avoids the introduction of estimated values of the molecular dimensions in Perrin-Einstein relations.

The equivalent viscosities obtained with 1,6-diphenyl hexatriene and with 2-pentyl-2′-butyl-4,4′-dimethyl oxazolidinoxyl are strikingly similar in egg lecithin vesicles above 0°C, while in dipalmitoylphosphatidylcholine dispersions above their transition temperature, a discrepancy of about 30% seems to remain, even at high temperatures.

Introduction

The use of viscosity to characterize bilayers or membranes and the values of this parameter obtained with inserted extrinsic probes in such systems is often considered unreliable. The reasons for such uncertainty are summarized below:

A. Phenomenological grounds. A friction tensor cannot be defined in a medium of finite extension. Even if this difficulty is overcome, the 'mean'

viscosity in an anisotropic medium cannot be a single parameter, since the components of shear viscosity are the residual non-diagonal terms of the viscoelastic tensor.

B. Microscopic reasons. A continuum hypothesis is untenable in bilayers since the probe is smaller than the surrounding molecules. Moreover, the probe causes its environment to deform whenever its size is greater than the local free volume dimensions in the host medium [1].

Studying the motions of unbound nitroxides (for instance that of steroid derivatives) allows one to obtain the diffusion coefficient tensor components of these molecules in liquids [1,2], soap dispersions [3], phospholipid dispersions [4—11], lipid extracts [12] or membranes [13—16].

On the other hand, numerous reports on order and fluidity deduced from nitroxide motions were published during the last decade (see, for instance, Refs. 17—23. References on this literature can be found in various reviews [4,24].

However, in all these works, either the calculations are stopped after deriving the diffusion (or correlation time) tensor components, or the evaluation of the viscosities is performed using the Einstein-Perrin equations [3,5,25]. This latter procedure is not rigorous for anisotropic fluids and the problem of the viscosity remains.

This kind of derivation is still more tenuous when using covalently bound labels [25].

Recently, some works of another kind [26—32] gave the principal components of similar parameters in membrane models by mean of a fluorescent and almost rod-like molecule, the polarized emission of which is followed during the fluorescence decay. Here, also, the derivation of viscosity from the diffusion tensor components raises the same difficulties and the respective contributions of probe and membrane anisotropies to the depolarized decays are not yet completely clear.

C. Inhomogeneous probe distribution. The transverse gradient of order throughout the thickness of the membrane [18,33,34] definitely induces a fluidity gradient. It can also induce a concentration gradient for the solute. This will complicate the viscosity averaging and would become drastic when the local probe concentration must be known to estimate the viscosity (e.g., fluorescence quenching). On the other hand, the gradients can be averaged by using probes of sufficiently large size.

Secondly, phase separations or 'clusters' often occur in bilayers. They induce differences of solubility for the probe (microheterogeneity) [35,36]. Thirdly, interactions between the probe and particular components of the membranes are frequently observed; a few of them have been used to study specific membrane sites [37—40].

In summary, a few workers measured anisotropic friction components in very local and highly specific situations, while others generally looked at relative or qualitative variations of 'fluidity' on changing the parameters of their systems, despite all the foregoing objections. Consequently one might think that the search for quantitative 'mean' viscosities is neither valid nor interesting.

However, the question cannot be resolved so quickly: it is still important to

be able to compare results given by different workers who used various probes or labels, even if the anisotropic diffusion components remain the most significant physical properties when describing a very local situation. Consequently, a reference viscosity scale seems still useful, even if the mean values it gives are for the moment justified by semi-empirical considerations only.

For such a purpose, Shinitzky et al. [41—44] previously used the fluorescence data given by a rod-like molecule dissolved either in a reference oil or in membrane bilayers. Some of us later showed [45,46] that this method gives very different values of Shinitzky's 'microviscosity' when the molecular shape and weight of the reference aliphatic oil are changed. In the same way, we recently described the procedure which must be applied to the 'mean' diffusion coefficient of the rod-like probe in liquids of very different macroscopic viscosities with a view to obtaining consistent results.

Similarly, the aim of this paper is to show what reliance can be placed on such a generalized method: the chemical nature of the probes is extended here to an electron spin resonance probe (nitroxide), while the reference media are again a large variety of alkanes and esters.

The quantities we obtain are not 'micro' viscosities in the sense of the Einstein-Perrin equations, but they are the macroscopic viscosities of isotropic media which induce, at the same temperature, the same microscopic behaviour as does the studied bilayer. It seems to us that 'microviscosity' is more appropriate for the parameter occurring in the Einstein-Perrin equations while we will use the term of 'equivalent' viscosity for the above-defined semiempirical quantity.

For such a generalization and its validity, the equivalent viscosity must stay unchanged when different kinds of chemical probes, studied by different physical methods, are used. Such a procedure can meet the chemical objections since, for instance, there is no reason why each kind of molecule (aromatic fluorophores, aliphatic chained nitroxides, etc.) are located in sites of similar fluidity. The more numerous and more various are the probes, the more certain are the evaluations.

Our method was already able to predict the macroscopic viscosity of an unknown oil from the microscopic probe data, which was not the case with Shinitzky's simplified procedure [45,46]. We propose to extend this consistency to the results given by the bilayers and membranes, at least in their liquid-crystalline phase.

II. Materials and Methods

A. Chemicals

1. Mineral oils, glycerol esters, bis-(2-ethyl)-hexyl sebacate and hexadecane isomers came from the sources previously mentioned [45,46]. These compounds were purified following the previously described procedures until their residual fluorescence was less than 2% of that of the fluorophore solutions prepared from them (FICA 55 MK II spectrofluorometer).

2. Egg phosphatidylcholine was extracted and purified in our laboratory. It was dispersed by sonication in buffered solution (pH 7.5) and subsequent ultracentrifugation so that Huang type liposomes were formed [47,48].

L-α-Dipalmitoyl phosphatidylcholine (synthetic) was supplied by NBC and

was used without further purification. It was also dispersed into the buffer by simple stirring above the gel-liquid crystal transition temperature. This led to Bangham type liposomes or 'multibilayers' [48,49].

For electron spin resonance measurements, more concentrated dispersions of both these phospholipids (100 mg/ml) were sometimes necessary: they were prepared by preliminary sonication, as above. Then, if necessary, repetitive freezing and melting cycles between −30°C and +30°C gave multibilayers.

3. The 2-pentyl-2′-butyl-4,4′-dimethyl-3-oxazolidinoxyl ('5N10' in literature) was synthetized following Keana's procedure [50]. It was purified by repeated high-pressure liquid chromatography (Waters kit).

4. The 1,6-diphenyl hexatriene ('DPH') was from Aldrich and was used as supplied.

5. Insertion of the probes into the lipid phases was performed either by a preliminary colyophilization with the lipid before its hydration or by mixing a known quantity of evaporated probe solution for 3 h with the water/lipid system in its liquid-crystalline state.

B. Physical methods

1. The average frictional coefficient $\bar{f}^*$ of the fluorophore was deduced from the thermal variations of two optical quantities, obtained as described previously [42,51]:

(a) the fluorescence lifetime, τ, of the fluorophore;

(b) the anisotropy of fluorescence polarization, r, derived from the emitted parallel and perpendicular light intensities under steady-state illumination.

Then

$$\frac{1}{\bar{f}^*} = \frac{1}{kT} \cdot \frac{1}{6\tau} \cdot \left(\frac{r_0}{r} - 1\right) \tag{1}$$

where k is Boltzman's constant and r_0 is the value of r extrapolated to infinite viscosity.

2. The average frictional coefficient, $\bar{f}^{\uparrow}$, of the nitroxide was calculated from the heights and widths of the electron resonance hyperfine spectra. These were recorded on a Varian E 109 spectrometer (X-band, equipped with a Varian temperature control accessory).

a. Clearing the experimental spectrum of its 'probe in water' component. In the aliphatic liquids and esters used, the nitroxide has an homogeneous environment and the line heights and widths can be directly measured on the recorded spectra. The situation is different in lipid/water systems: in the absence of spin-spin interactions the spectrum is constituted by the sum of two triplets; one given by the nitroxide lying in water, the other by its molecules located in the aliphatic chains of the lipid.

To free the spectrum from the contribution due to the probe in water, we could choose from the procedures used by previous authors:

Choosing free nitroxides with low solubilities in water such as the cholestane spin label. Here, we preferred to use a smaller probe.

In the case of smaller nitroxides (more soluble in water), raising the lipid/water ratio. But in these cases, the lipid 'suspensions' often become pasty and sometimes even pellets have to be prepared [52]. This kind of sampling is

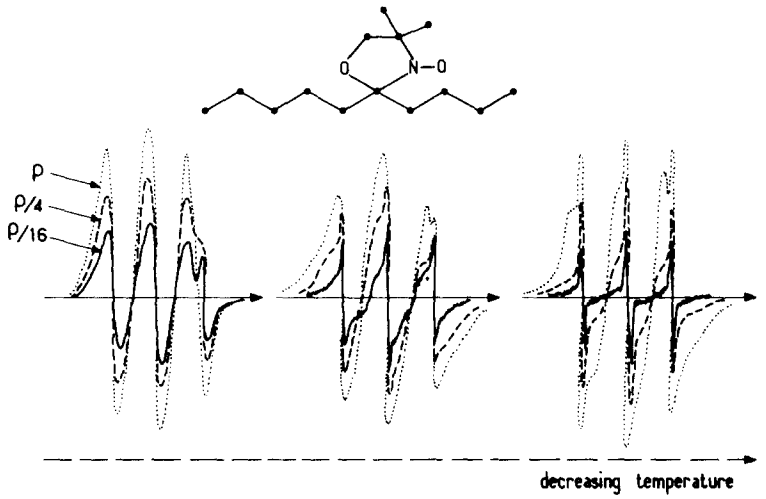


Fig. 1. Evolution of resonance spectra at fixed temperatures when changing the (lipid-water) ratio ρ. This allows combination of spectra by couples with a view to the elimination of the contribution of the probe in water. The 'pure aliphatic' contribution was isolated by subtracting one out of the other two experimental spectra corresponding to the same temperature (i.e.: all the thermodynamic parameters are kept unchanged, except for the (lipid/water ratio).

evidently inapplicable to rare materials such as those found biologically.

Quenching the paramagnetism of the nitroxide molecules in water by externally located paramagnetic ions [1]. This procedure, unfortunately, gives a simultaneous broadening of the spectral lines corresponding to the probe in lipid.

In numerous papers the partition coefficient was deduced from the partial splitting of the high-field line and was studied versus different thermodynamical parameters (see, for instance, Refs. 1, 5, 13, 36, 52, 53). Sometimes relaxation times have been measured, thanks to this partial splitting and despite the residual superposition of both the spectra: Either, the shape of the spectral lines given by the probe in lipid was approximately evaluated under the condition that the two mixed spectra exhibit lines of very different widths [19] (this needs adjustment by some examination of the relative intensities); or, each of the mixed spectra was separately computed by simulation procedures. Then the balanced calculated components were added [5,54].

In this work, we chose a nitroxide sufficiently small to 'insert' rather than to 'accomodate' itself [55] in the host lipidic chains (formula: cf. paragraph *A*3 above and Fig. 1). As a consequence of its size, this molecule is slightly soluble in water. For instance, if the surrounding chains are melted, the contributing spectrum of the probe in water is observed for lipid/water ratios in the range of 12 mg/ml and less.

As we wished to keep a procedure applicable to this range of lipid concentrations, it was necessary to be able to use the raw split spectra. We chose to compare the shapes of spectra lines given by concentrated lipid samples on the one hand, and those deduced from combining two spectra obtained from diluted samples on the other hand. For this purpose:

Firstly, concentrated systems (dispersions or vesicles, $C_l \geqslant 100$ mg/ml) were prepared: one might think that if the concentration were sufficiently high (nevertheless with complete hydration of polar heads), the probe-in-water contribution (compared with that of the probe-in-lipid one) would become negligible.

Secondly, we prepared either two other samples by successive dilutions to 1/5 of the preceding one, or another primary solution of approximately 12 mg/ml, then, from this, a second and a third one by successive dilutions. By this means, the nitroxide partition was modified while the overall nitroxide/lipid ratio was conserved (see spectra in Fig. 1).

For these last preparations, the spectrum corresponding to the probe in lipid was derived as follows: first we normalized the three experimental spectra by dividing each of them by the height $h_{aq}^{(-1)}$ of its own high-field line component which corresponded to the probe in water only (Fig. 2a); secondly, we subtracted one of the other two such normalized spectra and so we obtained the spectrum of the probe in lipid.

Evidently, the method requires that all the thermodynamic parameters other than the lipid/water ratio be kept constant when recording the three experimental spectra.

Digitization of the experimental spectra was performed with a Haromat (Hagen system) coordinatoscope and subsequent calculations were done with a PDP 11 computer (Digital Equipment). The subtracted spectra were recorded on a Benson drawer: they were analyzed as those coming from the Varian spectrometer (cf. paragraph *b*, below).

(b) Evaluation of relative correlation times. It must be emphasized that the relative values of the correlation times are sufficient for our purpose, even if they are not proportional to the 'actual' correlation times, τ_c. Consequently, various expressions could be taken for the relative 'τ_c'.

In the 'fluid' region, i.e., as long as its value was less than $6 \cdot 10^{-9}$ s, we evaluated this parameter by the Kivelson procedure [56]:

$$'\tau_c' = 1.27 \cdot 10^{-9} \cdot [\{h(0)/h(+1)\}^{1/2} + \{h(0)/h(-1)\}^{1/2} - 2] \cdot \Delta\mathcal{H}(0) \qquad (2)$$

where $h(m)$ = heights of the triplet lines ($m = +1, 0, -1$); $\Delta\mathcal{H}(0)$ = widths of the central line in G ('τ_c' in s)

In this range of correlation times, the value of τ_c is nearly independent of the diffusion model [57]. Consequently, the rotational diffusion constant can be evaluated by $(1/6\tau_c)$ by a continuum model as well as by a jump diffusion theory. Therefore the reciprocal of the average frictional coefficient of the nitroxide was taken as:

$$\frac{1}{\bar{f}^{\uparrow}} = \frac{\tau_c^{-1}}{6kT} \qquad (3)$$

However, the use of the $D = 1/6\tau_c$ relationship lead us to consider $\bar{f}^{\uparrow}$ as an 'apparent' parameter for the time being.

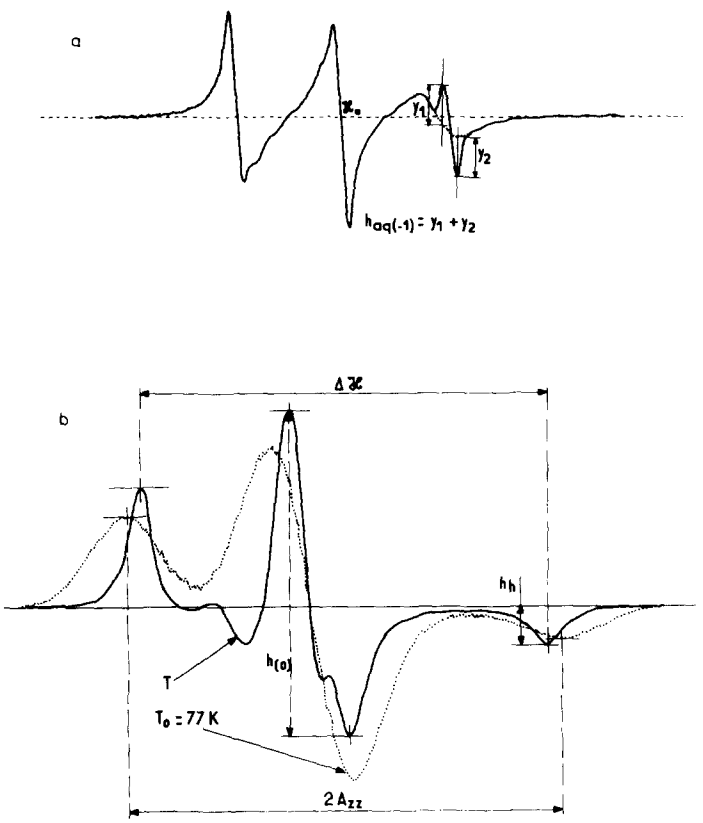
In the 'slow tumbling' region [7,8,57–62], an empirical relation was used. It was primarily derived following a formalism similar to that of Goldman [62, 57]. Then the $[h(0)/h_h]$ term was introduced (Fig. 2b) and the different coefficients were optimized to obtain the best continuity with the foregoing $1/\bar{f}^{\uparrow}$

Arrhenius plots (that being done for all the liquids, cf. Results, below):

$$\frac{1}{\bar{f}^{\uparrow}} = \frac{1}{6kT} \cdot \left[3.94 \cdot 10^{-5} \left\{ \frac{2A_{zz}}{2A_{zz} - \Delta\mathcal{H}} \right\}^4 \cdot (h(0)/h_h)^2 \right]^{-1} \tag{4}$$

In this range, the high-filed line of the nitroxide triplet has vanished while a wide and negative band replaces it at still higher field values ('strongly immobilized' state band or 'powder spectrum'). h_h is the height of this band and $\Delta\mathcal{H}$ is the overall width of the absorption spectrum (Fig. 2b).

There is no possible overlap between the ranges in which $1/\bar{f}^{\uparrow}$ is deduced either from relation 3 or from relation 4. It follows that a slight gap on the equivalent viscosity scale cannot be avoided. Fortunately this gap is very



Fg. 2. a. The height $h_{aq(-1)}$ of the contribution of the 'aqueous' nitroxide population is evaluated for two different lipid/water ratios, all other thermodynamic factors of the system being kept unchanged. Because the normalizing line is narrow, we assume it remains symmetrical in height and field throughout the temperature range investigated (2 to 75° C). For the first digitalization of the spectra, the dashed line was drawn approximately but in the subsequent computing program $h_{aq(-1)}$ was incremented by small quantities until a less noisy result was obtained. The normalization of both the spectra was done before their subtraction by multiplying each by the reciprocal of its own $h_{aq(-1)}$ value. b. Parameters measured in the 'slow tumbling' region for the derivation of $1/\bar{f}^{\uparrow}$ by relation 4. The widest spectrum shown is taken in polyisobutens II or IV (cf. Refs. 45, 46) at 77 K.

narrow and the continuous curvature of the viscosity vs. $(1/\bar{f}^{\dagger})$ plots allows accurate interpolation.

(c) Effects of the lipid/water ratio: determination of 'τ_c' values in water-containing systems. If heating above 35°C is avoided during the preparation of the samples, the successive 1/5 dilutions of a 100 mg/ml lipid solution lead to correlation times smaller than those measured on the primary solution: this is observed all along the temperature range studied. As examples: (a) the maximum 'τ_c' depression reaches 50% after the first 1/5 dilution of diplamitoylphosphatidylcholine dispersion around its transition temperature. (b) It is typically 10—20% after the first 1/5 dilution of the same lipidic system in its liquid-crystalline state (and after 1/5 dilution of vesicle solutions of egg phosphatidylcholine).

The observed narrowing of the triplet lines which causes this apparent decrease of 'τ_c' is due to the intensifying of the hidden narrow spectrum of the probe in water: this is confirmed by the fact that the 'τ_c' depression becomes smaller with increasing temperature (i.e., when the lipid/water partition coefficient rises). Finally the effect vanishes around 75°C.

Subtractions of normalized spectra corresponding to 25 and 8 mg/ml lipid solutions lead to the same values of 'τ_c' as initial concentrated systems: these values are kept as the correct ones. On the other hand, subtractions of normalized spectra corresponding to 8 and 2 mg/ml give values too small, in agreement with those directly measured with a 25 mg/ml solution.

Every time preheating has occurred during the preparation of the samples and in all cases above 40°C, nitroxide has been partially destroyed by contact with water (see Discussion below). This effect is mixed with the foregoing ones. When the effect appears, the measured correlation times deviate the other way: they are greater than the precedent, particularly for dilute solutions. These values must be severely controlled and are the most often discarded.

Under these conditions, the method becomes applicable to small quantities of lipidic materials.

Results

A. Thermal variations of the average frictional coefficient of the n-decane oxazolidinoxyl derivative (5N10) in alcanes, esters and aliphatic oils

Fig. 3a shows these variations over a range of viscosities extending from 1 to $2 \cdot 10^6$ cp. Similar results have been obtained with 1,6-diphenylhexatriene as a probe (Fig. 3b and Ref. 46). The sequence of solvents was the same in Figs. 3a and b for the probes: except inside the C_{16} isomer group, no inversion appears in the classification order of the liquids used. Moreover, this order is the same as that which appeared from the macroscopic viscosities [45,46].

B. Consistency of the average frictional coefficients of both the probes in liquids

Microscopic data on the motion of both the probes in all the solvents studied lead to a single and direct relationship between their respective frictional coefficients (Fig. 4). This result could perhaps be expected, but the fact that these data are obtained by means of two different physical techniques allows us to

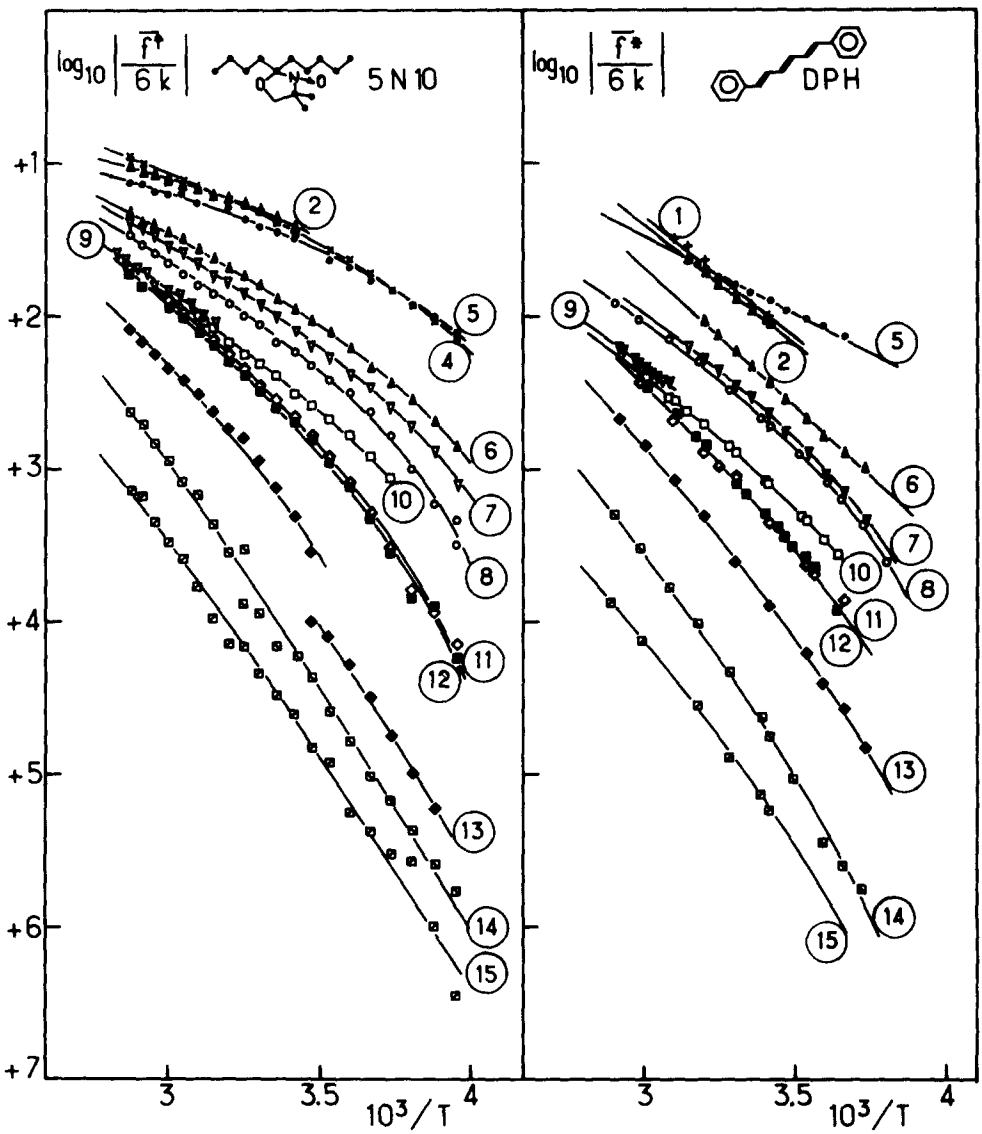


Fig. 3. Comparison between the thermal variations of the frictional coefficients $\bar{f}^{\uparrow}$ and $\bar{f}^{*}$ obtained from the nitroxide and the fluorescent probe in the same solvents as in Ref. 46. Both $\bar{f}/k$ terms are expressed in K · ns. ($\bar{f}^{*}$ is given by relation 1 (in text). For $\bar{f}^{\uparrow}$, measurement becomes relative when τ_c is more than 6 ns (see text). + (1) *n*-pentadecane; △ (2) *n*-hexadecane; □ (3) 4-methyl pentadecane; X (4) 6-pentyl undecane; ○ (5) 2,2,4,4,6,8,8-Heptamethylnonane; ▲ (6) glycerol tributyrin; ▽ (7) bis-(2-ethyl—hexyl sebacate; ○ (8) squalane; ▼ (9) glycerol trilaurin; □ (10) glycerol triolein; ◇ (11) mineral oil Primol 342; ■ (12) mineral oil USP 35; ◆ (13) cargille oil type B; □ (14) polyisobutene type II; □ (15) polyisobutene type IV; (1) and (3) were not studied with the paramagnetic probe while in fluorescence (3) and (4) are not pictured.

consider that the averaging of $\bar{f}$ remains valid at very high viscosities, at least in liquids.

C. Comparison between the values of $\bar{f}$ in liquids and the macroscopic data: principle of determination of an equivalent viscosity

To evaluate the 'equivalent' viscosities of unknown media from microscopic

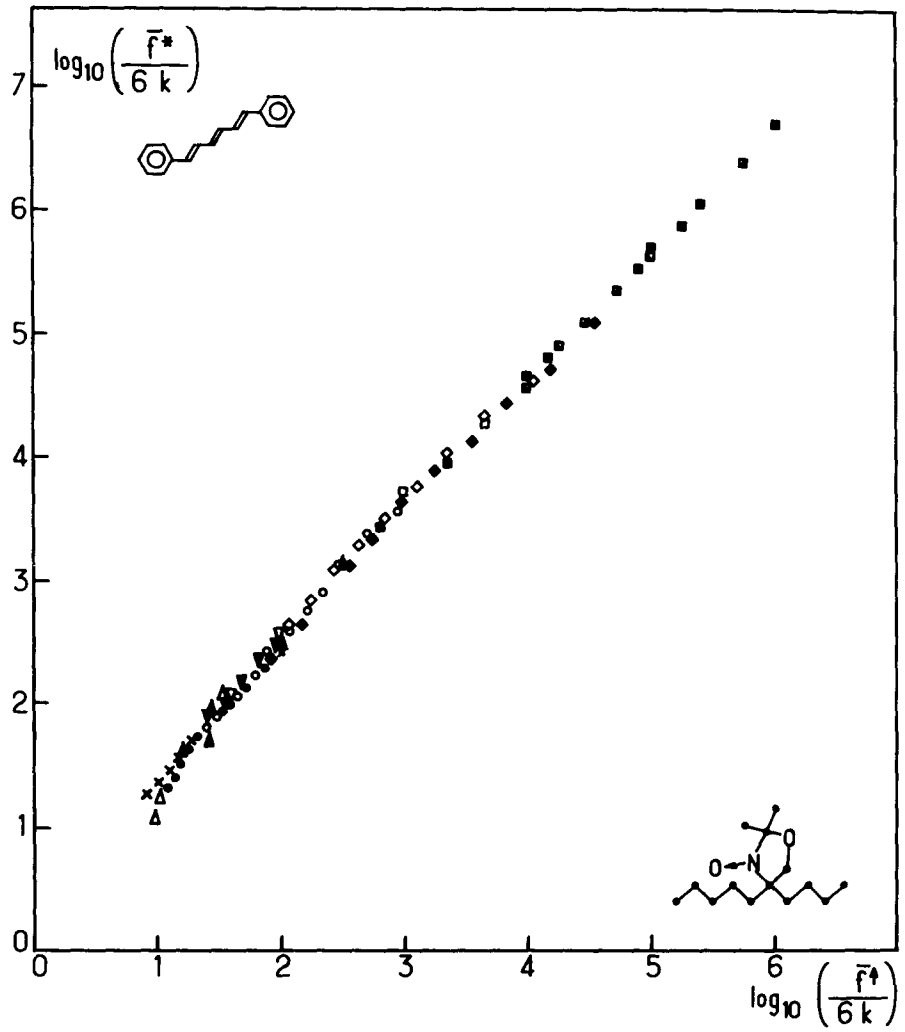


Fig. 4. Concomitant variations of the mean frictional coefficients of 1,6-diphenyl hexatriene and of 2-pentyl-2-butyl-4,4'-dimethyl-3-oxazolidinoxyl in the oils used in Fig. 3 (for symbols and numbers, see Fig. 3). For a given oil at every temperature, $\bar{f}^{\uparrow}$ and $\bar{f}^*$ were obtained from Figs. 3a and 3b, respectively.

determination of $\bar{f}^{\uparrow}$ or $\bar{f}^*$, the method we previously described [46] for the fluorophore was applied to the nitroxide. Fig. 5a gives the result of such an attempt: one obtained the relationships between the macroscopic viscosities and the above-defined parameter $\bar{f}^{\uparrow}$. Each liquid gives a curve different from the others (as when the rod-like fluorophore was used). To deduce the macroscopic viscosity of a given liquid from the measure of the probe motion therein with the help of standards, the only method is to draw, here again [46], the lattice of isotherms across the foregoing curves (Figs. 5a and b). When measured values of $\log(\bar{f}/6k)$ are taken for the abscissa, the temperature of the experimental determines the correct reference isotherm in the lattice and the equivalent viscosity can be read on the ordinate axis.

The fact that all the above results are consistent must be emphasized again: since Fig. 4 depicts a bijective relationship between the abscissa of Fig. 5a and that of Fig. 5b, each liquid will give the same equivalent viscosity upon using one probe or the other.

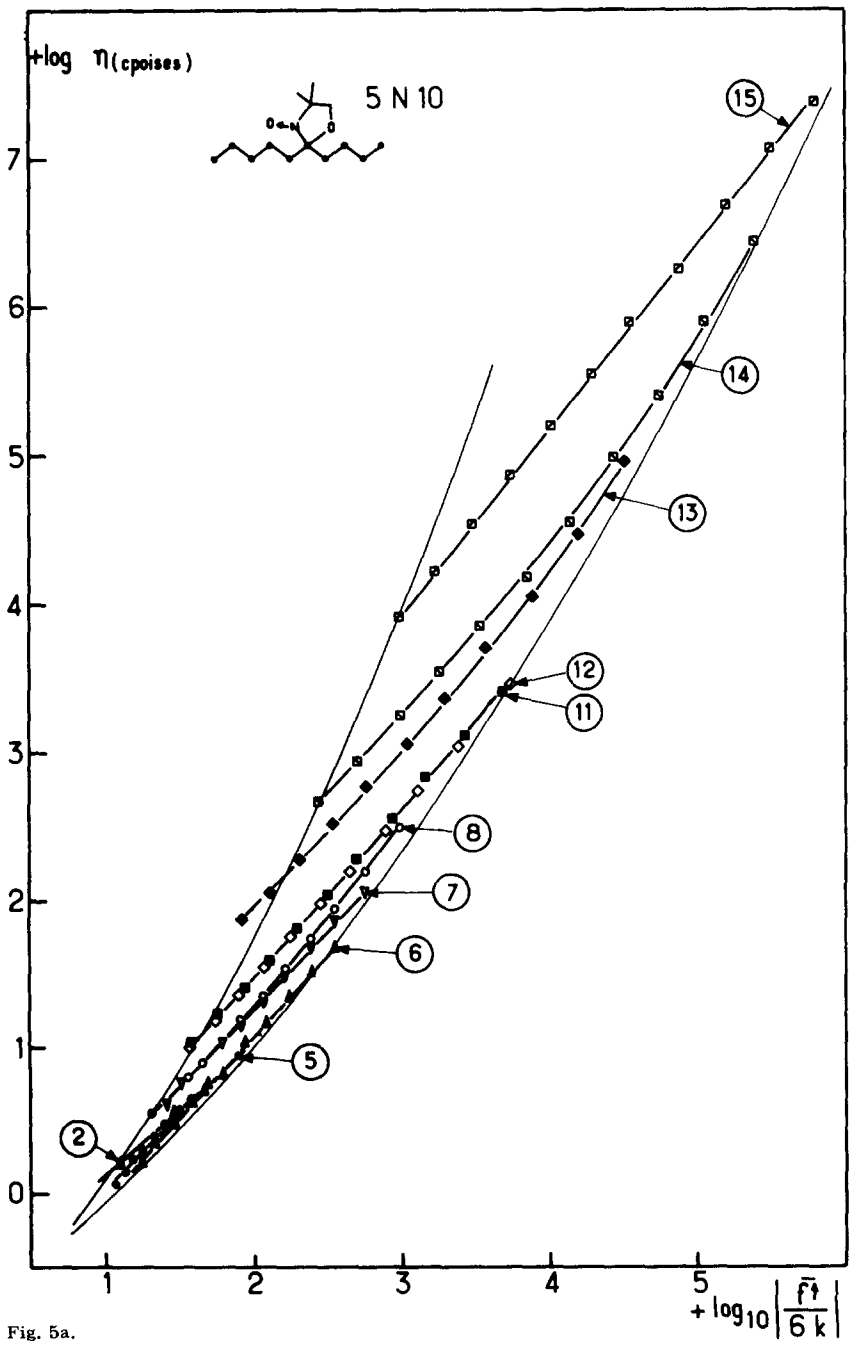


Fig. 5a.

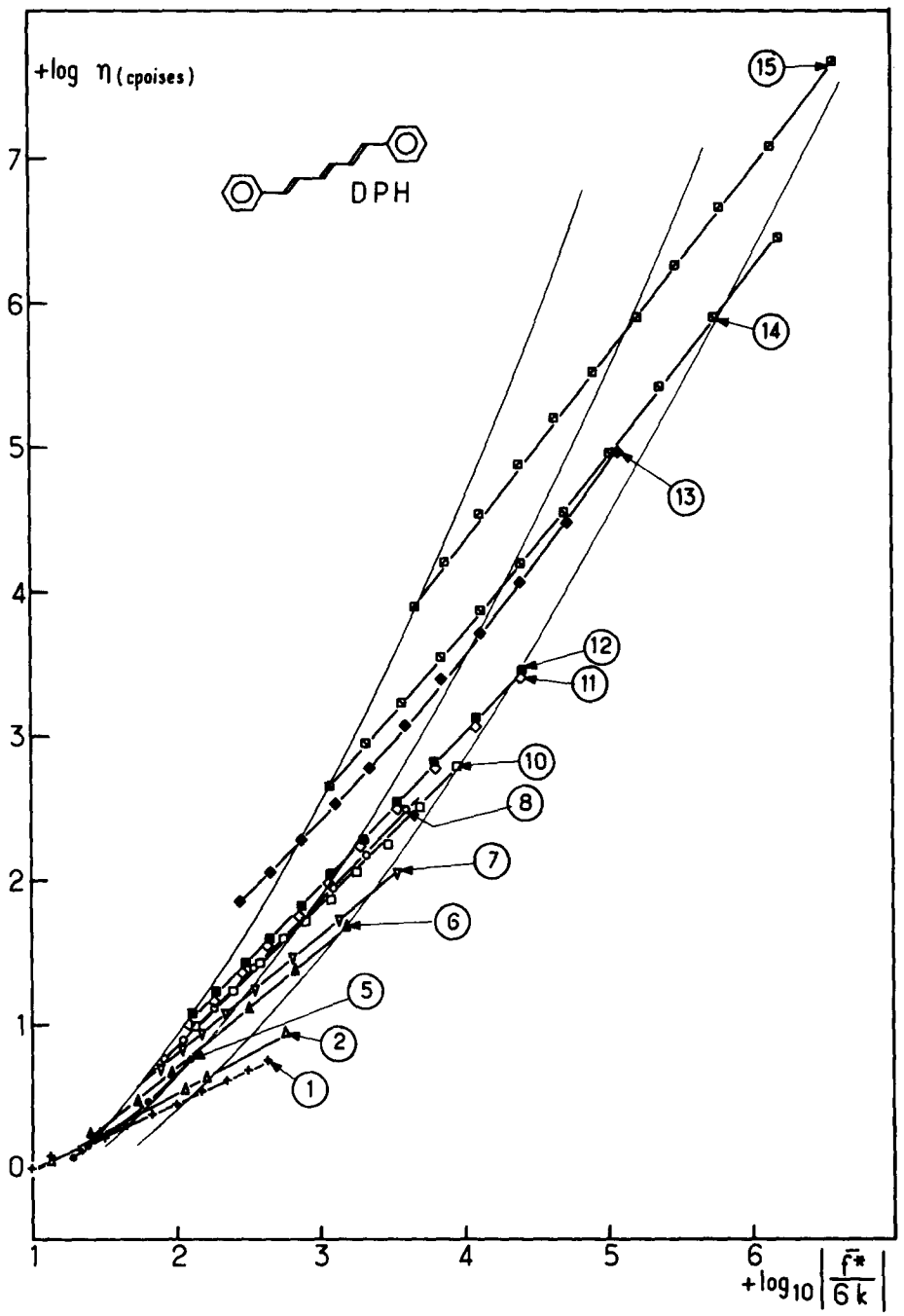


Fig. 5. Comparison between the log-log plots corresponding to a Shinitzky generalized procedure either with the nitroxide or with the fluorescent probe used here. For each temperature a different 'reference' curve is obtained (see Ref. 46). This result was not foreseen by previous authors, who used a single standard liquid. Here, similar lattices of isotherms are obtained with fluorescent and nitroxide probes.

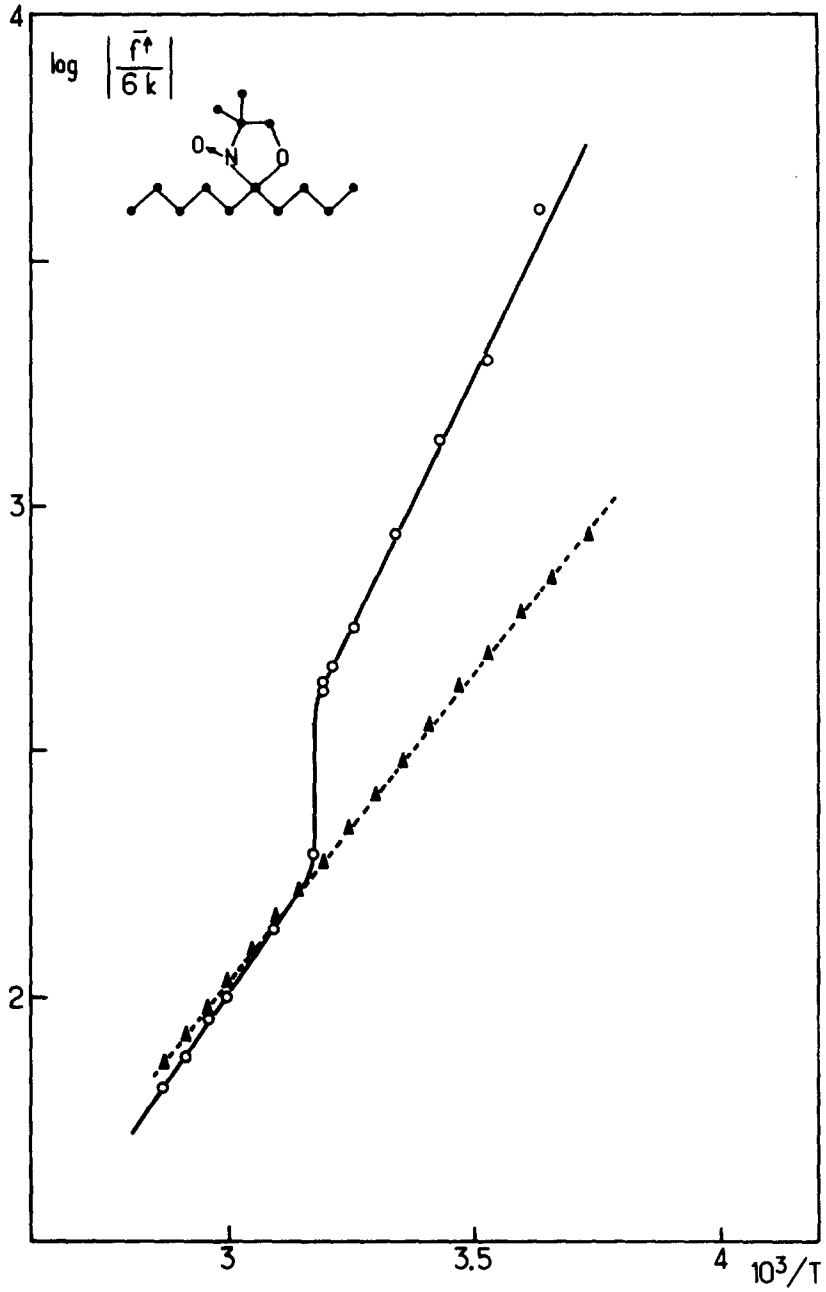


Fig. 6. Thermal variation of the mean frictional coefficient of 2-pentyl-2-butyl-4,4′-dimethyl-3-oxazolidinoxyl in the lipid systems studied. ▲, egg phosphatidylcholine (vesicles); ○, dipalmitoyl phosphatidylcholine (disperions).

D. Consistency of the $\bar{f}$ values given by both the probes in the liquid crystalline state of the phospholipids studied

The thermal variation of microscopic EPR data for both the phospholipids is given in Fig. 6.

We wished to determine whether each couple of $\bar{f}^*$ and $\bar{f}^\uparrow$ measured under the same conditions in lipids obeyed the relationship expressed by the curve of Fig. 4. For this purpose, the procedures of paragraphs *B* and *C* above were

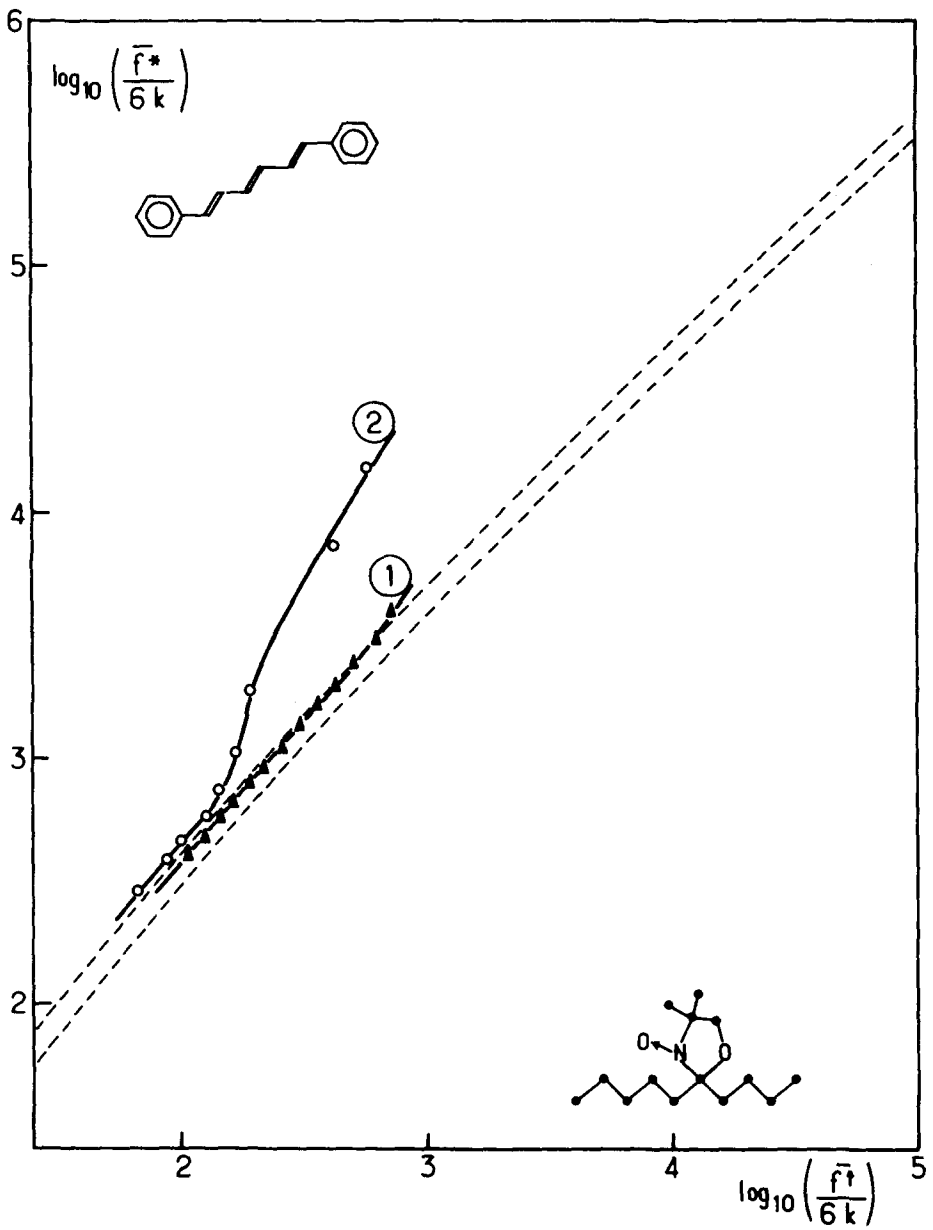


Fig. 7. Concomitant variations of the frictional coefficients of the fluorescent and nitroxide probe of Fig. 3 and Fig. 5 in egg phosphatidylcholine vesicles and in dipalmitoyl phosphatidylcholine multibilayers. The temperature was eliminated between Fig. 6 and the corresponding Arrhenius fluorescence plot (not given). For this last sample the variation involves the whole gel-liquid crystal transition. Dashed lines, extreme positions of the curve of Fig. 4. Lipid concentrations: 125 and 25 mg/ml for spin resonance measurement; 1 mg/ml for fluorescence measurement. ▲, egg phosphatidylcholine (vesicles: 2 to 70°C); ○, dipalmitoyl phosphatidylcholine (dispersions: 2 to 75°C).

applied to egg lecithin vesicles and to dipalmitoylphosphatidylcholine dispersions; i.e., the absolute temperature term was eliminated between Fig. 6 and the corresponding Arrhenius fluorescence plot of $\bar{f}^*/6k$, (not given). The result is shown in Fig. 7.

As long as the egg phosphatidylcholine system stays at least at 7°C above its chain transition, the values of $\bar{f}^*$ and $\bar{f}^{\uparrow}$ strikingly verify the same relation as found for the isotropic liquids. The uncertainty is less than 10% (0.04 on $\log_{10}$ discrepancies). This falls within experimental error. In egg phosphatidylcholine, the results cover a range corresponding to a maximal ratio of 50 between the extreme $\bar{f}$ values. The divergence is greater (approx. 30%) for the dipalmitoyl lecithin system in liquid state, even at high temperature. It must be emphasized that it is not the value of 'viscosity' which induces the deviation from the isotropic behaviour, but the proximity of the transition (compare curves 1 and 2

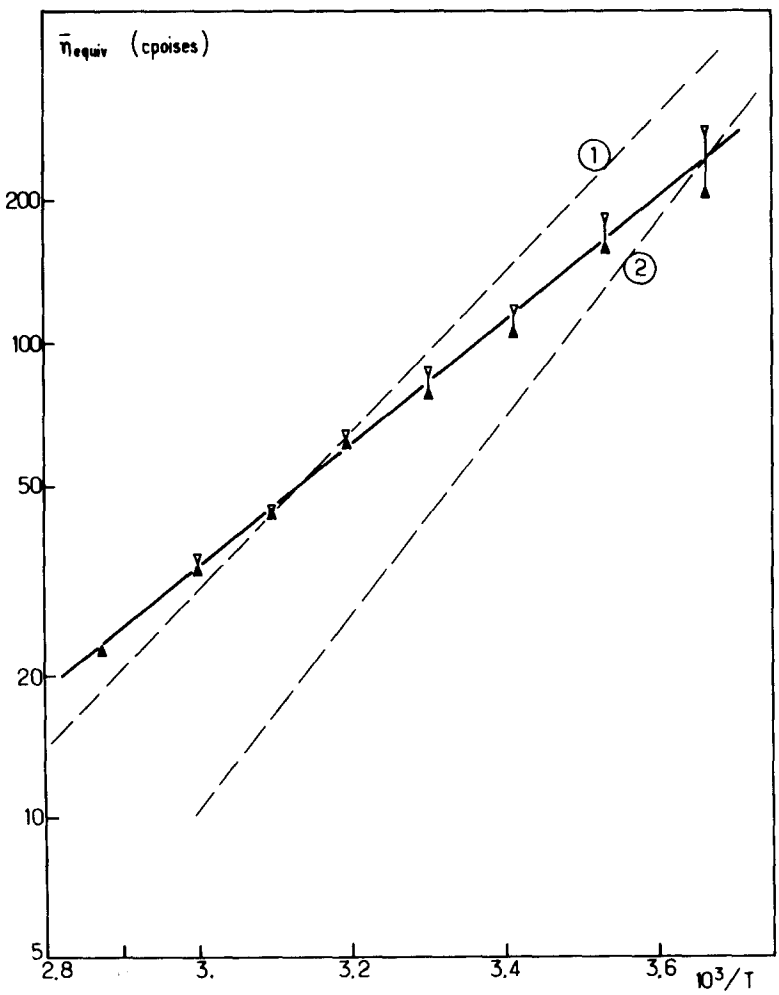


Fig. 8a.

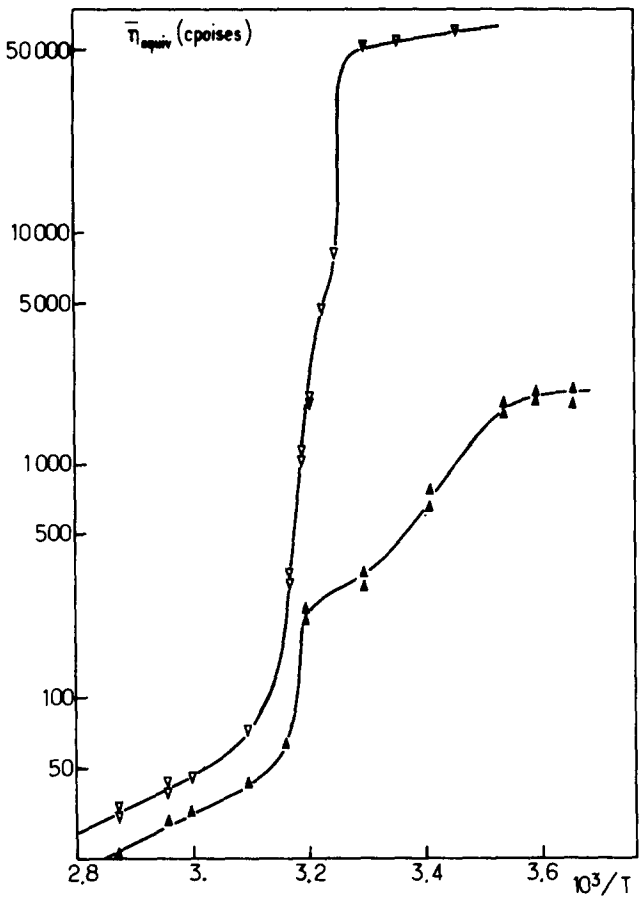


Fig. 8. Thermal variations of both the equivalent viscosities obtained by the method described: a, egg phosphatidylcholine; b, dipalmitoyl phosphatidylcholine. Full line (this work): ▲, From 2-pentyl-2-butyl-4,4'-dimethyl-3-oxazolidinoxyl motion ($\bar{\eta}^{\uparrow}$); ▽, from 1,6-diphenylhexatriene motion ($\bar{\eta}^{*}$). Dashed lines: (1) results from Cogan et al. [42] (perylene); (2) results from Shinitzky et al. [43] (1,6-diphenyl hexatriene).

in Fig. 7). This suggests that in a lipid bilayer, the anisotropy of the 'solvent' environment remains much greater than in the standard liquids, and is of a specific type.

E. Deduced values of equivalent viscosities

It becomes necessary to restrain the application range of the previously claimed 'microviscosities' (see for example Refs. 42—44, 46), to that over which the couples ($\bar{f}^{*}$, $\bar{f}^{\uparrow}$) are consistent with their isotropic relationship. On this condition, the use of equivalent viscosities, as defined in paragraph *C* above, is still justified. Application of the method to both the studied lipids is shown in Figs. 8a and b where the viscosities are termed $\bar{\eta}^{*}$ and $\bar{\eta}^{\uparrow}$.

There is no more reason to extend quantitative evaluation of a mean viscosity to the neighbourhood of the transition temperature, and still less below. In these ranges, only separated evaluations of the anisotropic components of fric-

tion coefficients are significant. Further, the use of Einstein-Perrin relations to each of these components remains unjustified.

It must be emphasized that by our procedure applied to egg lecithin we obtain an agreement between the two methods which is better than that previously obtained from two different fluorophores [42,43] or from the nitroxides used previously.

Moreover, the value of activation energy is here 6.02 kcal/mol. Its value is smaller than that previously published [4,42,43].

For the dipalmitoylphosphatidylcholine/water system (Fig. 8b), the discrepancy is rather high even in the liquid crystalline state, as could be foreseen from Fig. 7 (cf. paragraph *D* above).

However, the use of the 'equivalent' viscosity seems advantageous for this last system: it leads to an emphasis of the pretransition which was not detectable on the 'τ_c' Arrhenius plot (compare curve 2 of Figs. 7 and 8b).

Discussion

(1) Before presenting the principal objections concerning the localizations of the probes and their probable effects, we will emphasize some difficulties which are too often underestimated when doing thermal variations with nitroxides in aqueous systems and which will become dramatic for computing of simulated spectra.

(a) Lability of the nitroxide in the presence of water

The intensities of the three spectral lines decrease as a function of time in buffered solutions as well in solutions containing organic chemicals, as in phosphate buffer containing antireducing agents (see Table I, note d, for the compositions of buffer). This does not arise from the presence of nitroxide impurities, since no side-band was detected on high pressure chromatograms during the purification procedure. This decrease is accompanied by no detectable line broadening. Table I gives the relative variations of the middle-field line intensity $h(0)$ under various conditions, either in the presence of water or not: addition of unlabelled oil over labelled buffer firstly causes the spectrum the nitroxide in oil to appear, while the spectrum of probe in water simultaneously decreases; however, this extraction is accompanied by a diminution of the number of observable paramagnetic centers. This decrease is observed also by making contact between a labelled oil and an unlabelled buffer solution. On the other hand, when labelled oils are kept from contact with buffers, they do not show any decrease. One can conclude that the rapid decay of intensities in labelled buffer alone cannot be explained simply by a demixing of the probe *. Moreover, the compared time dependance of biphasic oil/buffer and lipid/buffer systems agrees with the complete disappearance of spin resonance in a diluted lipid/buffer sample kept at room temperature for 5 days.

* Detailed experiments on optical and EPR behaviours of nitroxide/buffer systems around and above the critical micellar concentration were performed after questions from the reviewers, and results are available. These experiments show that the 5N10/buffer solutions here described have concentrations very near or below the critical micellar concentration.

TABLE I

COMPARED LABILITIES OF THE 5-*n*-DECANE OXAZOLIDINOXYL DERIVATIVE (5N10) UNDER VARIOUS CONDITIONS

System studied (mono- or biphasic)	Lipid/water ratio (mg/ml)	Temperature (°C)	Residual intensities of nitroxide spectra (%) at the following times											
			minutes								days			
			8	12	16	20	24	40	60	120	1	5	8	240
Buffer, pH 7.5 [d]		room						50		0				
		75	80	71	65	50			0					
Bis-(2-ethyl-hexyl)sebacate		room					100							93
		75					100					81		
Sebacate over the buffer [d]		75 [a]	100								5	0		
Mineral oil Primol 342 over the buffer [d]		75 [b]	88	83	80	78.5	76					0		
Egg lecithin vesicles (buffered) [c]	100	room	99.5								31		0	
		65	76	46	25					2		0		
	20	room	99								0			
		65	76	46						0				
	4	room	90							0				
		65	76							0				
Dipalmitoylphosphatidylcholine dispersions (buffered)	100	room										80		
		75										0		
	20	room										30		
		75	90	80	75							0		
	4	room										0		
		75										0		

[a] Probe originally located in oil: spectrum of probe in oil only observable.

[b] Probe originally located in buffer: rapid decrease of the high field line corresponding to the probe in water and increase of the probe in lipid one (extraction).

[c] Nitroxide concentration: about 1 mol per 100 mol of lipid.

[d] With a view to excluding any possible destruction of the nitroxide by organic reducing compounds, we systematically compared the given times of disappearance between both the following buffers: (1) phosphate: $2 \cdot 10^{-2}$ M (pH 7.5) potassium ferricyanide: 0.34 g/l; (2) Tris(hydroxymethyl)amino ethane: $2 \cdot 10^{-2}$ M (pH 7.5) ethylene diamine tetraacetate; $2 \cdot 10^{-3}$ M. No difference was observed between the buffers.

(*b*) In a 2 mg/ml lipid sample with a 1/100 nitroxide/lipid molar ratio, the decay of intensities is so fast above 55°C that it occurs during recording: the middle- to high-field lines ratio $h(-1)/h(0)$ is the most affected and leads to greatly overestimated correlation times. The fast chemical destruction of the nitroxide in the buffer probably displaces the partition equilibrium so that the probe in water can no longer be detected and so that the spectrum corresponding to the probe in lipid decreases by subsequent diffusion. On the other hand, eventual broadening by exchanges with dissolved O_2 molecules can be prevented [54].

To conclude, we must emphasize that, until now, this artifact has rarely been outlined, but some works in which partially water-soluble oxazolidine oxyl derivatives of *n*-alcanes were used mention thermal effects [5] which could perhaps be explained by the present observations. Evidently, the accurate study of line shapes would be rather venturous in cases where such systems are not rigorously controlled.

We were led to study the evolution of spectra with time and with the lipid water ratios (paragraph III*D* above) with a view to the elimination of all possible causes of deviation. This was achieved by avoiding too great a dilution of the nitroxide and by comparing the values given at various lipid/water ratios.

Objections concerning the localization of the nitroxide can be met by the results of Sillerud and Barnett [63]: these authors showed that even for a rather hydrosoluble nitroxide (2,2′6,6′-tetramethyl piperidine *N*-oxyl or 'TEMPO') the probe fraction solubilized by the lipid is located at aliphatic chain depth and is not near the polar heads [19]; it seems that this will be the case, and with still more reason, for the probe used here.

The observed difference between EPR equivalent viscosity, $\bar{\eta}^{\uparrow}$, given by the gel and the liquid-crystalline states of dipalmitoylphosphatidylcholine is small when compared with the corresponding difference of $\bar{\eta}^{*}$. Since the reason cannot be imputed to unexpected nitroxide distribution, it becomes possible to invoke the partial destruction of the chain order by the guest molecule below the phase transition: ". . . besides the steric deformation, the Van der Waals forces between the aliphatic chains are broken by the polar NO group without any possible compensation" [25]. With such an assumption, the evaluated $\bar{\eta}^{\uparrow}$ values below the transition are too low: the measured quantities refer to unaligned aliphatic chains and to a subsequent local residual disorder.

While various models of molecular diffusion can be used to explain the nitroxide motions as long as the surrounded medium remains sufficiently fluid [57], in highly viscous states the probe molecules with sizes in the same range as those of the solvent (or still smaller) do not longer obey Brownian diffusion and require the jump diffusion model [57].

However, in this report the problem of choosing a diffusion model is minimized by the fact that only relative measurements are made by way of comparison between various solvents and lipids: moreover, the probe molecular dimensions are less than those of either the solvent or the lipid.

In another respect, it may be noted that in the case of egg phosphatidylcholine the inserted nitroxide apparently conserves an isotropic motion in the 'slow tumbling' region (in Fig. 7 the last five points concerning this lipid and corresponding to high $\bar{f}^{\uparrow}$ values (+20°C to 0°C) lie in the 'slow tumbling

region'). On the other hand, the discrepancy (approx. 30%) between the values of $\bar{\eta}^*$ and $\bar{\eta}^\uparrow$ in the dipalmitoylphosphatidylcholine system greatly reduces the physical meaning of the average microviscosity in lipids which contain only long saturated aliphatic chains.

It is probable that the observed motions of both the probes are essentially rotations or tumbling: consequently the described approach needs to be further confirmed by dynamic studies of another kind: those done on translational motions could be suitable since information they give will be different from that of the present study.

With these reservations, the use of an average viscosity may still hold, but only above the transition temperature of lipids.

Acknowledgments

We thank Dr. J.F. Faucon and Dr. P. Delhaes for fruitful discussions on the present problem. We are grateful to Dr. C. Garrigou-Lagrange who contributed to our knowledge of nitroxides. We thank Professor Prud'Homme and his colleagues from geology-oceanography Laboratory for their help with computing procedures. We are indebted to Mrs. E. Dupart and to Mrs. J. Favede for their careful technical assistance.

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