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VARIATIONS IN MICROVISCOSITY VALUES INDUCED BY DIFFERENT ROTATIONAL BEHAVIOUR OF FLUORESCENT PROBES IN SOME ALIPHATIC ENVIRONMENTS

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Summary

Recently there have been large developments in the indirect estimation of phospholipid bilayer and membrane microviscosities through the use of fluorescent probes with the help of paraffin oils as standard solvents.

Before applying this semi-empirical method to membrane systems, it seemed necessary to test: first, a large variety of probes (this has been done by many authors) and secondly, a large variety of aliphatic oils (there is little literature on these tests).

The present paper shows the variations of the rotational relaxation rates of three probes in relation to the viscosities of some aliphatic oils. When changing the oil but keeping constant the macroscopic viscosity, large differences appear in the relaxation rates of a given fluorphore (a ratio of 30/1 is observed in the extreme cases).

The microviscosities of membranes deduced from the probe motion will consequently exhibit large uncertainty, as is shown with dipalmitoyl phosphatidylcholine.

The cause of these different behaviours must be looked for in the properties of the oil. Particularly, the anisotropy of the solute-solvent interaction in the site where the probe is located depends in part on the internal order of the solvent which is used as a reference.

Introduction

Microviscosity is usually a parameter calculated from rotational or from translational diffusion coefficients by using the molecular dimensions of the diffusing species.

In the case of membrane bilayers, Shinitzky et al. [1—4] gave an alternative but empirical definition of the microviscosity: they considered a viscous liquid

as a reference, the molecular anisotropy of which is assumed to be identical to that of the membrane (e.g.: white oil USP 35); then they measured the fluorescence polarizations and lifetimes of different extrinsic molecular probes inserted in this medium. This allowed them to plot the rotational diffusion coefficient of the solute versus the macroscopic viscosity of this solvent; by this means, a reference curve was obtained for the given probe. Finally, the "equivalent" microviscosity of an unknown medium can be obtained through two different measures: the fluorescence polarization of the chromophore and its emission lifetime.

The choice of a viscous saturated aliphatic hydrocarbon as a reference medium seems to be justified by the chemical structure of some membrane bilayers. However, various molecular anisotropies are found in different saturated aliphatic hydrocarbons an the choice of "white oil" could be seem rather arbitrary.

We show in this paper that the "reference curve" of the previous authors has no privileged character. It is perhaps usable for some melted bilayers, but it is a very rough approximation for others and surely no reference at all for the rigid "solid" state of the aliphatic chains.

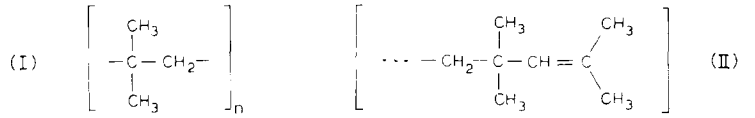
Elsewhere, Vanderkool et al. [5,6] also used another viscous aliphatic oil as a reference medium for an evaluation of the microviscosity from the translational diffusion coefficient of pyrene in membranes (Cargille-B immersion oil).

As will be seen in this paper, the anisotropic rotational behaviour of two fluorescent probes in the above mentioned oils and in many other is different enough to conclude that no convenient "reference medium" can be actually proposed for indirect determination of the membrane bilayer microviscosity.

Materials and Methods

(1) The following products were used without further purification: (a) Two synthetic short chain polyisobutenes (PIB II and IV) and natural white oil fraction Primol 342. They were gifts from Naphtachimie (13 Martigues, Lavera, France) and ESSO Research Laboratory (76 Mont St. Aignan, France), respectively.

The two first polymers are aliphatic hydrocarbons, which have structure (I):



(I) (II)

The initial group of these chains is a *tert*-butyl residue, but their tails generally allow an ethylenic bond (II).

Because its position is quasi terminal, one can think that this bond has no large effect on the chain conformation: this hypothesis is confirmed by depolarized Rayleigh scattering data (i.e.: the values of the so-called "optic molecular anisotropy excess"; this parameter is defined in refs. 7—10).

The molecular weights of these compounds are approx. 1000 for PIB II and 2900 for PIB IV. This leads to the following average number of carbons for their principal chain: C_{36} (PIB II); C_{100} (PIB IV).

Although their polydispersity is rather high (1.5—2), they are much better

defined compounds than the natural white oil fractions (most often, mixtures of alicyclic alcanes) such as our Primol or the USP 35 of the previous authors.

(b) Glycerol triolein from Fluka (puriss. grade) was supplied and always manipulated under dry nitrogen.

(c) The fluorescent probes: diphenylhexatriene from Aldrich Chem. Comp. (Milwaukee, Wisc.) and perylene from Fluka (purum grade). Preliminary experiments showed that successive sublimations of the latter did not change the measured values of the emitted light polarization.

(2) For the other products, further treatments were necessary. Squalane (from Prolabo, Paris) as well as the natural white oil fraction USP 35 (a gift from Am. O. Co., Whiting. Ind.) were purified by the following procedure.

(a) The solution of the studied hydrocarbon in cyclohexane (for ultraviolet spectrophotometry) was shaken with pure sulphuric acid (pro analysi). After decantation, the upper organic phase was treated similarly many times until the colour of the sulphuric phase became sufficiently light.

(b) After an aqueous washing, the cyclohexane solution was filtered through activated coal (Acticarbone from C.E.C.A., Paris). This solution was then concentrated under vacuum in a rotatory evaporator.

We found that the immersion oil for microscopy Cargille B (from Art. Thomas Co. Philadelphia, Pa.) did not resist the sulphonation and its solution in cyclohexane needs milder conditions: firstly, dilution of sulphuric acid with 80% H_3PO_4; then after decantation, the organic phase was washed with N/2 alcaline solution, shaken again with sulphuric $KMnO_4$ in acetic acid as a solvent and a minimum amount of water. Finally, a last washing with a N/2 NaOH solution was followed, before the coal treatment, by a filtration through a three inch column of acidic alumin., Kiesegel Merck n°C/60; 70—230 mesh, astm.

Each of these purification steps was checked by absorption and emission spectrofluorimetry (Cary 16 and Fica 55 M II) until the emission of the purified oil became negligible compared with that of the probe solution (at the concentrations used for polarization and lifetime measurements, i.e., generally $5 \cdot 10^{-7}$—$5 \cdot 10^{-5}$ M).

(3) The fluorescent molecule of benzo-(a)-pyrene exhibits a high photosensitivity and needs also some purification: the cyclohexane solution of the supplied material (from Fluka, puriss. grade) was filtered several times through Acticarbone, then evaporated.

(4) The calibrated vesicles (liposomes) of dipalmitoyl phosphatidylcholine were prepared in buffer solution from an analytical grade product (NBCo α-DL-phosphatidylcholine, β,γ-dipalmytoyl phosphatidylcholine (synthetic)) by sonication, ultracentrifugation and filtration on Sepharose 4B following the usual procedure described elsewhere [11].

(5) The viscosity measurements were made either with a Poiseuille-flow horizontal device, or with a rolling-ball viscometer. In their range of measurement, the values obtained for the same oil differ by less than 2% from each other. The Newtonian behaviour of the most viscous fluids (PIB II and IV, Cargille oil) was also checked with a variable strain rotation viscometer (Rotovisco Gebruderhaake, Berlin). With these viscometric techniques, the used

shear rates lie in the range $10^{-3} - 10^{+3}$ s^{-1} and they always give the same values for the macroscopic viscosity.

(6) The optical devices we used were: first, an automatic polarization fluorometer gave quasi simultaneously the ratios $(I/\!/ / I_0)$ and $(I_\perp / I_0)$ of the fluorescence intensities of the two polarized components to a reference intensity I_0, proportional to that emitted by the exciting source [12]. This apparatus used a chopper and a single photomultiplier; interference filters for the excitation and absorption filters for the emission beams allowed us the perform studies at variable wavelengths.

These measures led to the polarization P and to the total intensity I: $P = (I/\!/ - I\perp)/(I/\!/ + I\perp)$; $I = I/\!/ + 2I\perp$ with standard deviations of $\Delta P \approx 10^{-3}$ (for P between 10^{-2} and 0.5) and $\Delta I \approx 5.10^{-2}$ for I (in the range of 10; I in arbitrary unit).

Secondly, the decay times τ were obtained without photon counting by using a nitrogen-laser excitation beam [13]. This light source works at a low energy level to avoid the phenomenon of saturation of the fluorescence in the sample. Two photomultipliers respectively received excitation and emission pulses with appropriate polarizations.

A Tektronix oscilloscope (350 MHz) and its camera recorded both the responses on the same polaroid photograph: fifteen laser shots led to a satisfactory accuracy (0.05 ns for τ = 3 ns).

The fluorescence lifetimes were measured at least at two different temperatures for each solution: the thermal variations of I gave the other values (cf. ref. 1).

From the thermal variations of P and τ in different experimental conditions, we calculated for each temperature (a) the fluorescence anisotropy r:

$$r = \frac{2}{3} \cdot \left[\frac{1}{\mathcal{P}} - \frac{1}{3} \right]^{-1}$$

(b) The fundamental fluorescence anisotropy r_0, which is the value of r when the chromophore population is motionless.

(c) An Experimental relaxation rate $\bar{R}_{exp}$ can then be evaluated:

$$\bar{R}_{exp} = \left(\frac{r_0}{r} - 1 \right) \cdot \frac{1}{6\tau} \tag{1}$$

Results

1. Principle of calculations from experimental values

It has been shown in previous works [3,14—19] that, when the fluorophore has a symmetry of revolution in the direction $\vec{Oz}$, and relaxes around its three principal axes with the following principal rates: R_z around $\vec{Oz}$ and $R_x = R_y$,

If the absorption momentum $\vec{A}$ is colinear with $\vec{Oz}$

$$R_{exp} = R_x = R_y \tag{2}$$

If $\vec{A}$ is perpendicular to $\vec{Oz}$, and if each principal rate is very small compared

with $[1/\tau]$ ("small rotations hypothesis"):

(a) $\bar{R}_{exp} = \left(\frac{R_z + R_x}{2}\right)$ when the absorption and emission momenta $\vec{A}$ and $\vec{E}$ are colinear ($r_0 = +0.4$)

(b) $\bar{R}_{exp} = R_z$ when the angle of the momenta is $\frac{\Pi}{2}$ ($r_0 = -0.2$)

(c) $\bar{R}_{exp} = R_x = R_y$ when the angle of the momenta is $\frac{\Pi}{4}$ ($r_0 = +0.1$) (3)

In all cases, the relation between this angle $\alpha = (\vec{A},\vec{E})$ and r_0 is given by $\cos^2\alpha = 1 + (5r_0)/3$.

We will point out two results which are contained, but not explicitly described by Weber, refs. 17–19. First, relation (2) can be obtained either with a prolate or with an oblate ellipsoïd of revolution; the only conditions is $\vec{A}$ must be along $\vec{Oz}$. Secondly, for relation (3c), the "small rotations hypothesis" is not necessary. The conditions "$\vec{A}$ along Ox" and "$\vec{E}$ in the bissector plane of $(\vec{Ox}, \vec{Oy})$" are sufficient.

The function which is plotted from the experimental values is in fact the ratio of $(6\ R_{exp})$ to the absolute temperature T, or:

$$y = \left(\frac{r_0}{r} - 1\right) \cdot \frac{1}{T\tau} \tag{4}$$

This is done because Stokes-Einstein's value of $\bar{R}_{exp}$ for a sphere of volume V in Weber's theory is: $\bar{R}_{exp} = R_x = R_y = R_z = kT/6\eta V$. Consequently, for a sphere, y is proportional to $(1/\eta)$, and $\bar{R}_{exp}$ is not; furthermore:

$$y = K \cdot \frac{1}{\eta} \tag{5}$$

with $K = k/V$.

It is well known that for rod-like molecules such as diphenylhexatriene [3,4,20] the plots of y versus $(1/\eta)$ give also linear relations; with such a shape, the value of K is also simply related to the volume V : $K' = \frac{3}{4}K = \frac{3}{4}k/V$ [15]. For the disk-shaped molecule Perylene, it is experimentally difficult to use relations (3b) and (3c) above 0°C since, in these, r_0 is always small. If moreover η becomes too small, the corresponding values of r decrease so much that their measures are difficult to perform. *

On the other hand, the mixed effects of the two principal rates when $(\vec{A},\vec{E}) = 0$ (relation 3a) lead to a more complicated situation. The plotting of function y versus $(1/\eta)$ gives lines of various cuvatures, except when the in-plane relaxation rate R_z is much faster than the out-of-plane ones, R_x and R_y (ref. 19, partially used in Fig. 1).

Some attempts have been done (1) to introduce three "principal" viscosities in the equations of motion. This point will not be further developed here since η is not a true tensorial parameter and because Perrin's relations for the ellipsoïd become very complicated with two (or three) different viscosities. But

* At approx. 0°C, Shinitzky et al. [1,2] have however estimated R_z and $R_x = R_y$ by using the relations (3b), (3c) (cf. Discussion).

as long as the medium is considered isotropic, there can be no theoretical reason to find different values of K for the same fluorophore.

2. Anisotropy of microscopic motion in various aliphatic oils

It can be seen (Fig. 1) that for the USP 35/perylene mixture, our values of $y = f(1/\eta)$ are in very good agreement with the results of Cogan and Shinitzky [2]. The same agreement is found for our glycerol/perylene curve when compared with Weber's data [19] for the propylene glycol-perylene system. The last two solvents are closely similar environments for the probe. The small initial curvature can be interpreted either as an anisotropic but strongly privileged motion, or as an almost spherical behaviour of the probe embedded in a shell of solvent.

For the glycerol trioleine/perylene mixture, differences are observed between our experimental points and those given by previous authors [2]. But one of the most important results is the larger distance between these two sets of points and that given by the white oil/perylene solution.

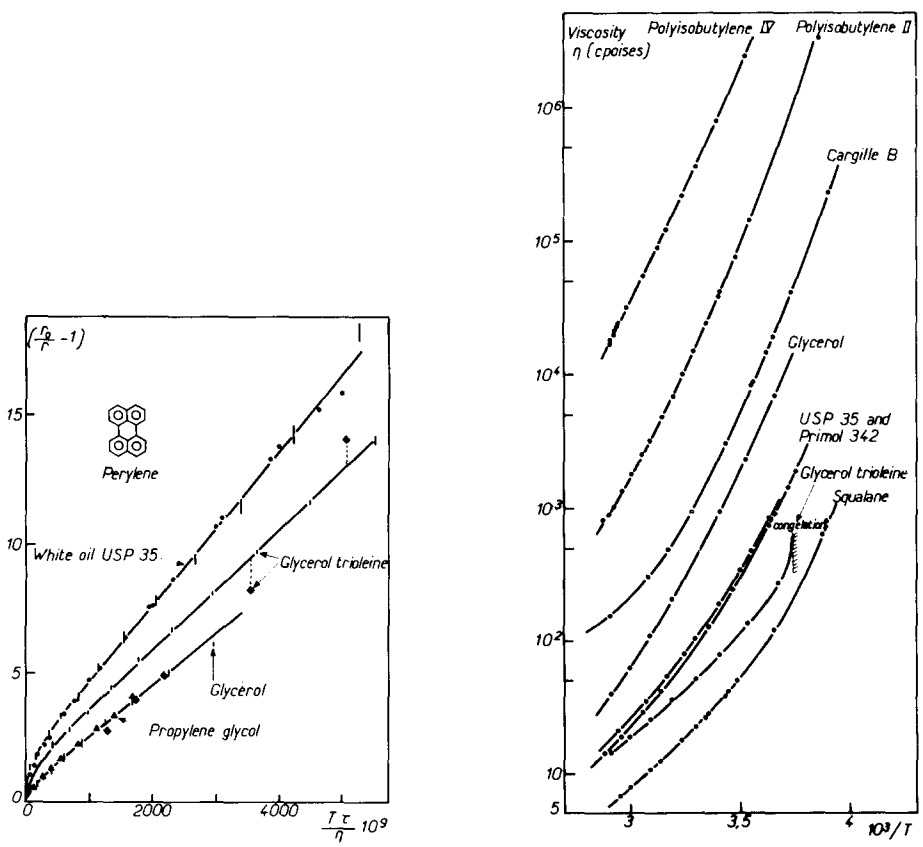


Fig. 1. Experimental agreement of present fluorescence and viscosity results with the data available in the literature: Weber [19]: ▲, propylene glycol. Cogan et al. [2]: ◆, glycerol trioleine; ●, white oil USP 35. Present results (with $[(1/\rho_0) - \frac{1}{3}] = 1.94$ at $\lambda_{excit.} = 367$ nm): |.

Fig. 2. Thermal viscosity variations (versus reciprocal absolute temperature $\times 10^3$) of various compounds used in this fluorescence study.

Consequently, we have systematically studied several saturated hydrocarbons of various structures (chain lengths and branching), the viscosities of which lie in a wide range even at room temperature (Fig. 2). Because one may use diphenylhexatriene to avoid the mixed effects of the different relaxation processes, as described above for the perylene, we have studied the same viscous solvents with these two probes and a few of them with benzo-(*a*)-pyrene (Fig. 3).

With diphenylhexatriene, we observe a very large dispersion in slope K of the y function (K PIB IV/K squalane ≈ 30). And when comparing the functions y obtained from the first two probes, we find, except for squalane, the same sequence for the solvents.

Furthermore, Fig. 4 shows that, when using diphenylhexatriene, the value of K is closely related to the value of η at constant temperature. The plot of log K versus is $\eta_{25^\circ C}$ gives the rough relation:

$$\log_{10} K \simeq 0.3 + 0.34 \log_{10} \eta_{25^\circ C} \iff K \simeq 2 \cdot [\eta_{25^\circ C}]^{1/3} \qquad (6)$$

Dispersed K values are not simply explained by chain length variation

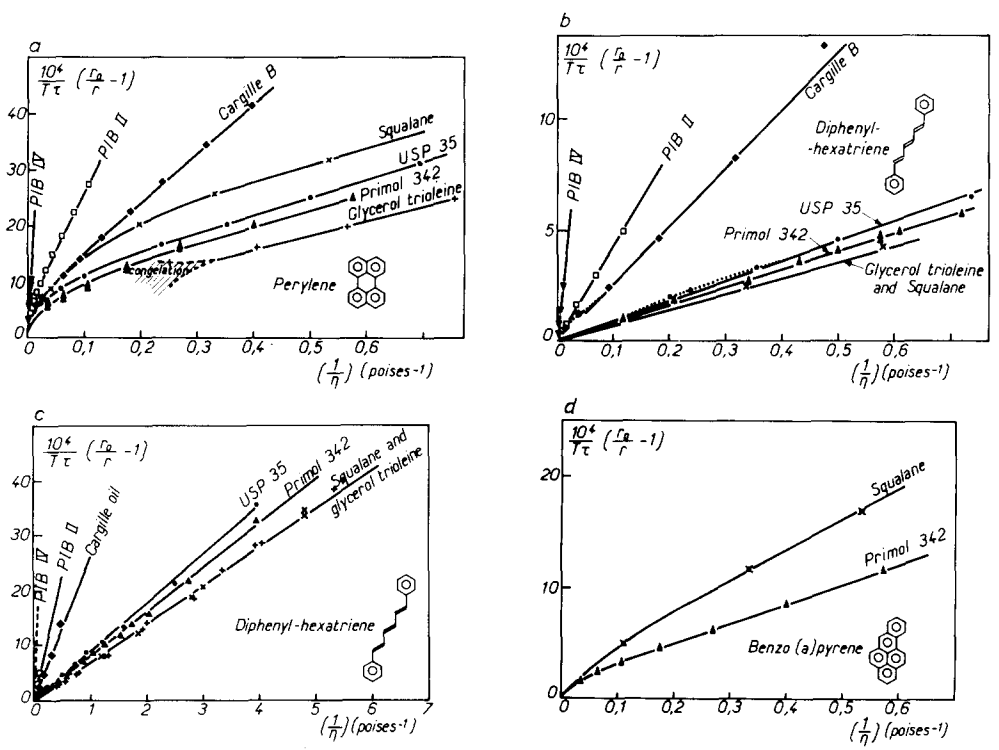


Fig. 3. (a) Perylene rotational behaviour versus the reciprocal microviscosity in the oils shown in Fig. 2. (b) and (c) Diphenylhexatriene rotational behaviour in the solvents of Fig. 2. Results are shown with two different scales to avoid less accurate log-log plotting. For each probe and each solvent the average number of experiments was 17 between 0 and 70°C (extreme experimental values from PIB IV and squalane could only appear on log-log plots). (d) Benzo-(*a*)-pyrene rotational behaviour in squalane and Primol 342. PIB IV: ▼; PIB II: □; Cargille B: ♦; USP 35: ●; Primol 342: ▲; Squalane: X; glycerol trioleine:

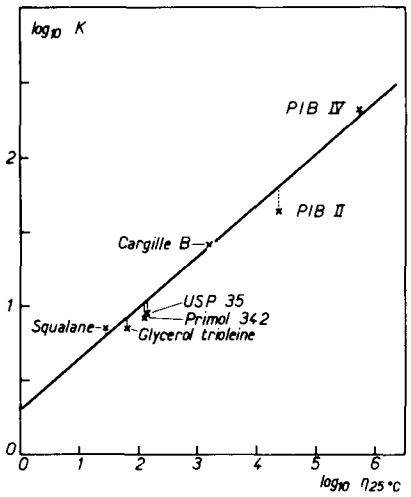


Fig. 4. Correlation between values of the slopes K given by diphenylhexatriene in the solvents of Fig. 3 and their viscosities at 25° C. Correlation is similar for other temperatures.

only (for example C_{36} and C_{34} principal chains in PIB II and squalane, respectively). Since, for a given probe, its shape is well defined, we must try to find the cause of the dispersed values of K in the various microscopic anisotropy of the solvents or of the location of the solute in these solvents.

Previous authors [1,2] have pointed out this difficulty and the insufficiency of Perrin's isotropic theory. However, they assumed a rather close similarity of anisotropy in various media when they proposed the method in order to evaluate the mean microviscosity [1,2].

A short-range order in hydrocarbons is still observed in the liquid state. Among the techniques which show such effects, there are either intrinsic methods in pure alkanes (viscosity [21], microcalorimetry [22] and depolarized Rayleigh [7]) or probe-using methods in membrane models (EPR [23]).

In binary solutions too, some of these techniques give evidence of hydrophobic interactions. For example, depolarized Rayleigh scattering and microcalorimetry allow to observe them between aromatic molecules and all-trans alkanes. These interactions perturb the local self order of the alkanes [8—10].

In aliphatic oils, depolarized Rayleigh scattering measurements lead to the conclusion that the short range order is much greater in the long chain hydrocarbons PIB II or PIB IV than in squalane or mineral oils *. (The analytic data on these last compounds, mass spectrometry, show that they are rather alicyclic than opened chain hydrocarbons.)

Consequently, with the help of depolarized Rayleigh scattering, the following interpretation of the results obtained here may be given qualitatively.

It must firstly be noted that the largest values of K are observed in liquids which, according to depolarized Rayleigh scattering, are the most ordered (e.g. PIB IV).

Let us now suppose that some short living organized aggregates do exist in the studied liquids. Following the above statement, such "clusters" are more

* Maelstaf, P., personal communication.

probable in the oils which exhibit large values of K. Moreover, if one assumes that the probe molecules are pushed off the clusters, they evidently return in an unorganized environment and no difference in fluorescence can be observed between the oils. Consequently if the liquid hydrocarbon contains such aggregates, the probe molecules are inside, at least in part.

Then one can note that, when increasing viscosity by decreasing temperature-reducing translational and rotational freedom of the solvent molecules, the all-trans conformations of the hydrocarbon chains become simultaneously more probable. The intermolecular distances between these microscopic parallel "rods" become evidently smaller, but it is not impossible that plate-like or rod-like molecules of probe could move relatively faster in such an environment than in a disordered one which would have the same viscosity (for the probability diphenylhexatriene molecules to be in line with the chains of a membrane system, see ref. 15).

3. Consequences on the estimated microviscosities of membranes

Because of the dispersed values of the "reference" y function, differences in the estimated microviscosities of membranes must be expected. For this reason, we measured again the well known phase transition of the dipalmitoyl phosphatidylcholine vesicles by using the three above mentioned probes.

Fig. 5 shows the values of log η versus the reciprocal of the absolute tempe-

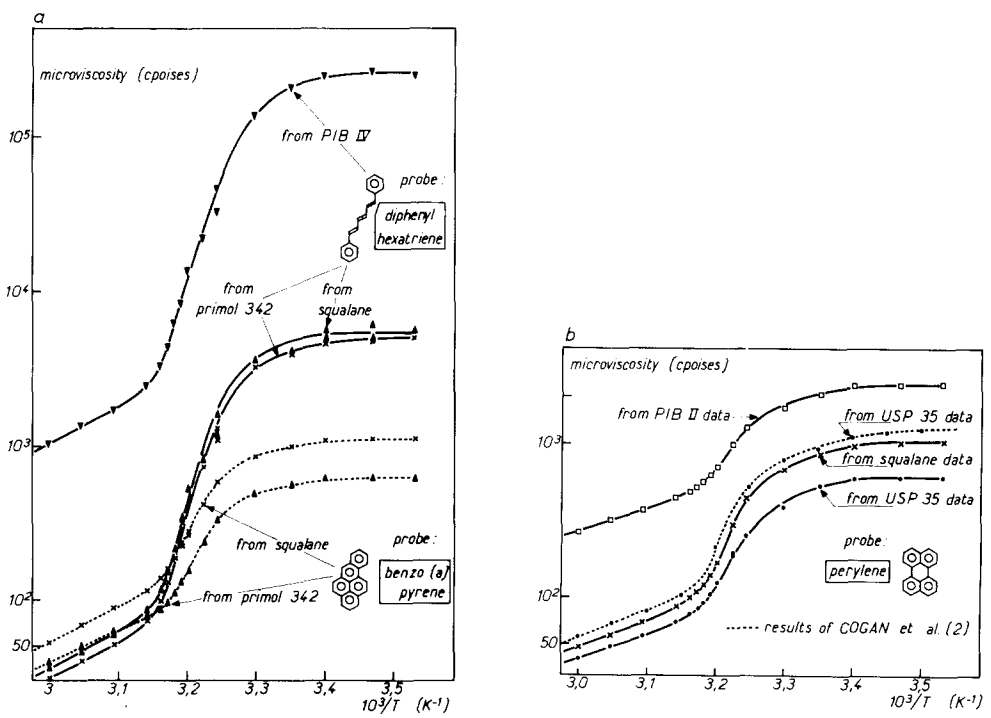


Fig. 5. Variations in microviscosities values of dipalmitoyl phosphatidylcholine obtained when using three probes and different oils as references. (a) with diphenylhexatriene and benzo-(*a*)-pyrene; (b) with perylene.

rature. One can seen that for a given reference solvent, the various probes give also different microviscosities. Only in the melted phase, the results are less dispersed (cf. also egg phosphatidylcholine in ref. 2).

When comparing the possible states of the lipid bilayer with our series of hydrocarbons, one may assume that, when a probe is labelling the gel state (i.e. the most "packed"), a rather rough model for this system would be the probe/PIB IV solution. On the contrary, for the liquid-crystalline state, the model systems could be solutions such as probe/squalane or probe/white oil.

However, even with the help of the results presented in this paper, it is difficult to choose the correct references for one or the other of the physical states of the bilayer. This is the reason why we used the above "reference" curves in their whole range each time.

So, even if the relative variations of microviscosity in a given membrane as a function of its thermodynamic parameters are still significant, comparing such values from one membrane to another seems irrelevant at the present time.

Discussion

The rotational anisotropy of a solute molecule can be conceptually separated into two uncorrelated parts: first, the anisotropy of the site where this probe is located; secondly, the lack of sphericity of the relaxing molecule. Such a decorrelation is not so obvious. It seem difficult to solve this problem of anisotropy with non-spherical probes which necessarily introduce their own anisotropy.

By other means, Shinitzky et al. [1,2] have shown how the variations of the fundamental polarization $\mathcal{P}_0 = 3r_0/(2 + r_0)$ versus the excitation wavelength and the concomitant variations of r allow an estimation of the ratio of the two principal relaxation rates in various media by using the relations (3b) and (3c). This ratio is a measure of the rotational anisotropy. They concluded that this parameter is almost the same in the assumed isotropic media (e.g. propylene glycol/glycerol mixture at 4°C, ref. 1, or propylene glycol at −14°C, ref. 2), in aqueous micelle solutions or quaternary ammonium salts as well as in lysolecithin micelles or in egg phosphatidylcholine [2].

Using this method, they observed a significant change in the values of $(R_z/R_x) = (R_z/R_y)$ after cholesterol insertions only. So, they assumed that in egg phosphatidylcholine without cholesterol, the probes were located far enough from the polar heads to reflect an unorganized internal structure.

In this way, they justified the use of "unorganized" white oil as a good calibration solvent for microviscosity measurements.

Our results first show that large differences appear even between saturated hydrocarbons, while in the previously proposed method, "it was assumed that when a dye molecule has the same degree of polarization in two different environments (the reference white oil and a lipid dispersion or micelle . . .), the effective molecular rotational volumes . . ." i.e. the reciprocal of the shape factor K ". . . in the two solvents are identical" [2].

Secondly, they indicate that separating the anisotropic rotational relaxation in two parts (the shape of the dissolved molecule and the solvent viscosity) according to relation (5) is not relevant since the "shape" contribution

$K = 6r_i/(T/\eta)$ (with i = x, y, z) still depends on specific solvent properties.

Our next purpose in this subject is to check this point by achieving depolarized Rayleigh scattering measurements on all the above studied hydrocarbons in order to investigate whether or not some quantitative correlations can be found between the short range organization in these liquid and parameter K defined here.

Since the scattering by lipid dispersions does not allow depolarized Rayleigh scattering method to be applied to the studies of membranes, other techniques will be necessary to eventually justify the possible usefulness of fluorescent probes for the evaluations of absolute microviscosities.

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