LIPID FLUIDITY OF RED CELL MEMBRANES ASSESSED WITH DIFFERENT FLUORESCENT PROBES

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SUMMARY

Two fluorescent probes, DPH and its cationic derivative, TMA-DPH, have been employed to probe the lipid fluidity of red cell membranes. The results show that the informations given by DPH and TMA-DPH can present important differences, suggesting that DPH and TMA-DPH are not localized in the same areas of cell membranes. In an attempt to investigate relations between lipid fluidity and rheological properties, the behavior of probes was studied in a Couette viscometer with a device for studying the emissive properties of probes when red cell membranes are under shear conditions. This report describes the system and provides preliminary results in employing the device.

INTRODUCTION

Important cellular functions such as rheological properties are regulated by membrane fluidity. An approach of fluidity can be made by using fluorescence polarization with fluorescent probes distributed in the lipid areas of membranes.[1-5] Changes in lipid fluidity can sometimes be related with either pathological processes or membrane alterations caused by cell interactions with exogenous molecules.[6] It is therefore important that the indications obtained regarding lipid fluidity should be accurately correlated with membrane structure or functions. However, biological membranes represent very heterogeneous media and the use of probes which label different compartments of cell membranes is required to get a better knowledge of membrane fluidity.

Derivatives of the classical probe: 1,6 diphenyl-1,3,5-hexatriene (DPH) are now available. Its cationic derivative: 1-4-(Trimethylamino) phenyl-6-phenylhexa-1,3,5-triene (TMA-DPH) has a moiety affixed to the para position of one of the phenyl ring.[7] Given their stuctures, the probes might be located in different regions of cell membranes. Thus, it is important to be able to compare data on these two fluorescent probes. In this paper, we present results obtained using TMA-DPH and DPH in red blood cell membrane, in particular the responses of probes to alterations in lipid cohesion caused by the introduction of cholesterol in the membrane. Additional investigations with an originally developed viscometer were carried out to study the response of probes embedded in deforming membranes in a fluid shear field.

MATERIALS AND METHODS

1. Preparation of erythrocyte membranes. 50 ml of blood collected on ACD were treated according to the method described by Heidrich and Leutner.[8] After several washings of hemolyzed cells, different experimental procedures were carried out to get «unsealed» and «resealed» ghosts. Unsealed ghosts were obtained by suspending membranes in 5 mM sodium phosphate buffer pH 8.0. As regards resealed ghosts, the membranes were treated according to the method described by Heven and Solomon [9] by adding a reversal solution followed by resealing at 37 °C for 1 hour. Membrane concentration was then estimated using a Lowry test.

2. Incorporation of cholesterol hemisuccinate. Cholesterol hemisuccinate was incorporated in erythrocyte membranes according to the technique described by Borochov and Shinitzky.[10] Red cell membranes (100 μg proteins/ml) were incubated with cholesterol hemisuccinate at room temperature for a period varying from one hour to 24 hours. After three washings in PBS, the protein concentration was adjusted to 100 μg/ml. Red cell membranes were incubated under the same conditions in a medium without cholesterol hemisuccinate. These suspensions were used for control purposes.

3. Fluorescent markers. DPH (Aldrich) and TMA-DPH (Molecular Probes) were dissolved in tetrahydrofuran (THF) and N, N-Dimethylformamide (DMF) respectively at a concentration of 2×10^{-3} M. For labelling, 3 μl of probe solution was directly added into 3 ml membrane suspensions. Final probe concentration was 2×10^{-6} M.

4. Instruments and polarization measurements. The assays were carried out using two systems developed in Photophysics Department of LA 328-CNRS (ENSIC-INPL, Nancy, France):

- a prototype of a continuous excitation fluorescence polarization instrument with the principle residing in a T assembly for simultaneously recording $I_{\parallel}$ and $I_{\perp}$. It is now being marketed under the name «Fluofluidimeter S1» (Sefam, Nancy, France);
- a «Couette» viscometer originally developed to perform fluorescence polarization determinations (Fig. 1). An interferential filter (Oriel) selected the excitation wavelength. On this

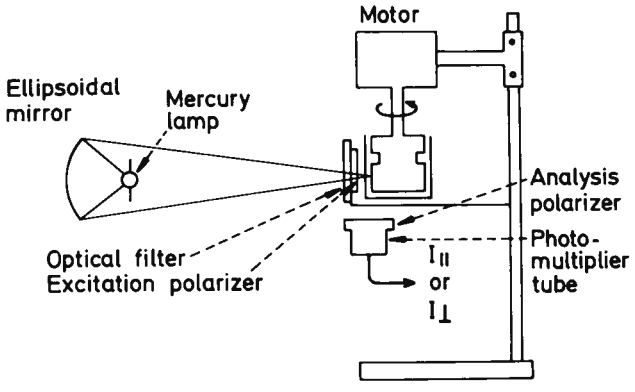

Figure 1: Schematic diagram of Couette viscometer for fluorescence polarizarion measurements.

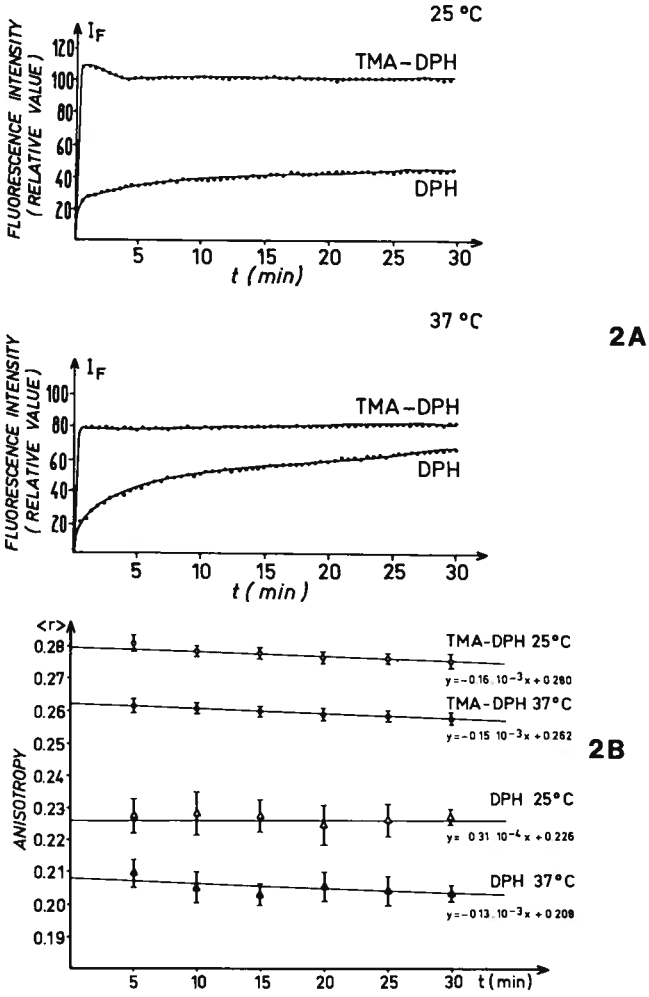

Figure 2: Time variation of fluorescence intensity (2A) and anisotropy (2B) of DPH and TMA-DPH during probe incorporation in erythrocyte membranes.

experimental device, the excitation was horizontally polarized. A quartz measurement cuvette was placed in a thermostatic holder. The inner cylinder was in Teflon. Parallel and perpendicular components of the fluorescence emission were alternatively recorded at 90° of the excitation through a filter and a polarizer.

Before each experiment, signals from samples of unlabelled cells or membrane supensions were recorded to determine correction factors. After determination of signals from «blank» samples $Ib_{\parallel}$ and $Ib_{\perp}$, fluorescence intensities of labelled samples were determined: $If_{\parallel}$ and $If_{\perp}$.

TABLE I **Fluorescence anisotropy of DPH and TMA-DPH embedded in erythrocyte resealed ghosts**

Probe	Anisotropy vs temperature 25 °C	37 °C
DPH (*a*)	0.238 ± 0,006 (*b*)	0.210 ± 0.003
TMA-DPH	0.270 ± 0.002	0.260 ± 0.001

(*a*) Erythrocyte membranes (100 µg/ml) were incubated for 15 minutes with DPH at 25 °C, 10 minutes with DPH at 37 °C and 10 minutes with TMA-DPH at both temperatures.
(*b*) Data represent the mean values ± standard error of 5 samples.

The results were expressed in terms of:

$$\text{steady state anisotropy } \langle r \rangle = \frac{(If_{\parallel} - Ib_{\parallel}) - (If_{\perp} - Ib_{\perp})}{(If_{\parallel} - Ib_{\parallel}) + 2\,(If_{\perp} - Ib_{\perp})}$$

and relative fluorescence intensity values:

$$IF = (If_{\parallel} - Ib_{\parallel}) + 2\,(If_{\perp} - Ib_{\perp})$$

RESULTS

1. Kinetics of DPH and TMA-DPH incorporation. The variations in fluorescence intensity and anisotropy were observed in DPH and TMA-DPH while the markers were being incorporated in erythrocyte membranes at 25 °C and 37 °C (Figs. 2A and 2B). The evolution of fluorescence intensity during the incorporation of fluorescent probes varies considerably with the marker. For TMA-DPH, fluorescence intensity increases very rapidly and in the minute following incorporation has already attained almost maximun value. In contrast, the evolution of fluorescence intensity is much slower in DPH. As regards the changes in anisotropy during marker incorporation, the two probes also behave differently. No significant changes were observed in DPH fluorescence anisotropy at 25 °C. In contrast, a reduction in anisotropy was observed in all the other cases. Because anisotropy could vary in some cases over a period of time comparison of results was carried out at a fixed time lapse after marker addition. Under strictly defined labeling conditions (15 min for DPH at 25 °C, 10 min for DPH at 37 °C and 10 min for TMA-DPH at both temperatures), TMA-DPH fluorescence anisotropy proved to be much greater than that of DPH (Table 1).

2. Influence of cholesterol hemisuccinate on TMA-DPH and DPH fluorescence anisotropy. the results given in Table 2 indicate that the two fluorescent markers respond differently to the introduction of cholesterol in erythrocyte membranes. A significant increase in DPH anisotropy in cholesterol enriched red cell membranes was observed. In contrast there was almost no change in TMA-DPH anisotropy.

3. Influence of a shear stress on the anisotropy of TMA-DPH and DPH embedded in «unsealed» and «resealed» ghosts. When «unsealed» ghosts were subjected to a linearly increasing shear field, a significant decrease in the anisotropy was recorded (Fig. 3A). For «resealed» ghosts, the decrease was weaker and was only observed when the determinations were performed within 5 hours after membrane preparation (Fig. 3B). The phenomena were reversible (Figs. 3C and 3D) and the results nearly similar for the two probes.

DISCUSSION

The results show that DPH and TMA-DPH behave differently when incorporated in erythrocyte membranes. The differences observed are probably due to the fact that the two probes are not located in the same site in the membrane. Indeed, although the location of DPH in the membrane has not been clearly defined, it can be assumed that the probe is situated in the hydrocarbon zone of the lipid bilayer.[11] As regard as TMA-DPH, some authors have put forward the hypothesis that the probe is located near the phospholipid polar head groups.[7, 12-15] The results reported in this paper appear to corroborate this hypothesis. TMA-DPH anisotropy is always higher than that of DPH, indicating that it characterizes a membrane area with a higher degree of order. However, the changes in anisotropy observed while the two probes were being incorporated call for comments on the hypothesis that TMA-DPH is anchored at the level of phospholipid polar heads. Apart from the results observed with DPH at 25 °C, when anisotropy does not change during probe incorporation, a low but systematic decrease is observed in all other cases. The variations may be related with the probes being slowly diffused from the outer zones of the membrane to the deep zones. Therefore, these results lend us to assume that the whole TMA-DPH molecules do not remain anchored at the lipid-water interface.

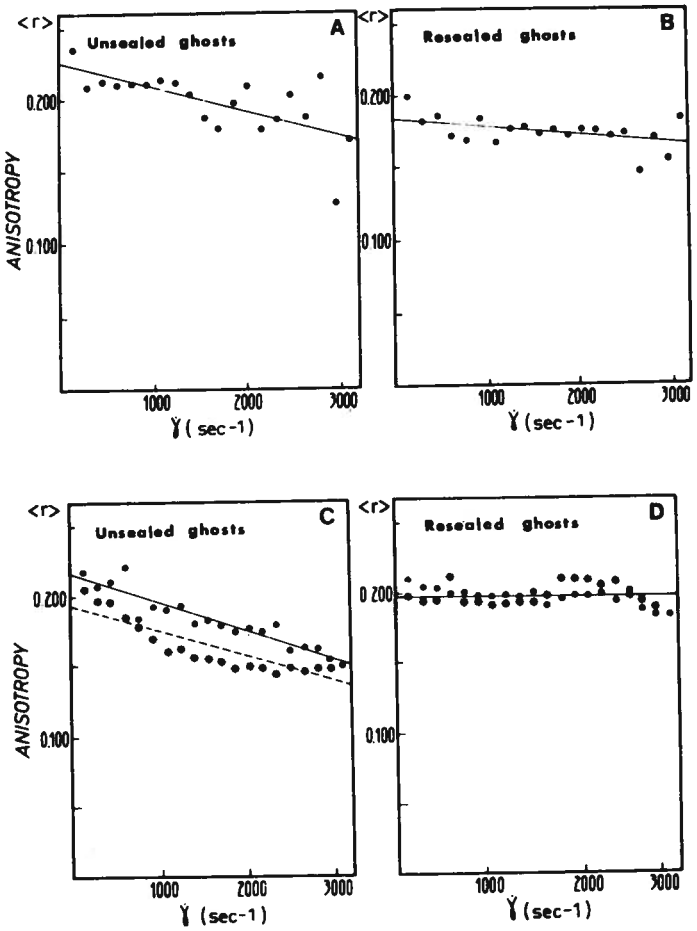

Figure 3: Effect of shear stress on the anisotropy of TMA-DPH embedded in unsealed (3A) and resealed (3B) erythrocyte ghosts. Reversibility: 3C and 3D.

The comparative study on the two probes also provides interesting informations on membrane changes caused by cholesterol. It has been reported that cholesterol rigidifies biological membranes.[10] Inasmuch as cholesterol partitions in the hydrophobic core of cell membrane, the absence of any changes in spectroscopic characteristics with TMA-DPH, therefore, tends to emphasize the possibility that cholesterol only rigidifies specific areas of cell membranes. However, it needs to be established that in the case of TMA-DPH, there is no significant variations of other spectroscopic parameters, i. e., lifetime. Whatever it may be, the results clearly show that the use of more than one fluorescent probe is required to report precisely fluidity changes occuring in cell membranes.

TABLE 2 **Effect of cholesteryl hemisuccinate on DPH and MA-DPH fluorescence anisotropy in erythrocyte resealed ghosts**

Incubation time with cholesteryl hemisuc. (hrs)	Anisotropy vs Membrane treatment Controls (ethanol)	+chol. hemi.	Variations in <r>(%)
	DPH		
1	0.253 ± 0.007 (a)	0.291 ± 0.005	+ 15.0
2	0.259 ± 0.003	0.284 ± 0.005	+ 9.6
4	0.243 ± 0.007	0.281 ± 0.004	+ 15.6
24	0.255 ± 0.006	0.292 ± 0.006	+ 14.5
	TMA-DPH		
1	0.273 ± 0.003	0.263 ± 0.002	— 3.6
2	0.277 ± 0.002	0.263 ± 0.003	— 5.0
4	0.263 ± 0.003	0.255 ± 0.002	— 3.0
24	0.272 ± 0.004	0.274 ± 0.005	+ 0.7

(a) Steady-state anisotropy assays were carried out at 25 °C after a time lapse for marker incorporation of 15 min. for DPH and 10 min. for TMA-DPH.

In contrast, the behavior of TMA-DPH and DPH are nearly identical when they are embedded in erythrocyte ghosts submitted to a shear stress, suggesting that the whole compartments of cell membranes are affected. The most striking result was the finding that significant changes in the anisotropy of fluorescent probes are recorded with unsealed ghosts and not with resealed ghosts. It may be suggested that the application of a linearly increasing shear field to unsealed ghosts induces a progressive decrease in the membrane cohesion. Whether the evolution in fluorescence anisotropy is linked to a higher deformability of unsealed ghosts compared to resealed ghosts needs to be clearly demonstrated. However, it is possible that the presence of holes in unsealed ghosts [16] results in higher deformability. In the same way, the weaker effect of shear stress on resealed ghosts with membrane ageing could be the consequence of a deformability loss with time, as reported for intact red cells.[17]

Further studies are needed before concluding to relations between deformability and fluidity. At present, the studies described here show the applicability of fluorescence polarization method for estimating fluidity and the usefulness of such a technique for studying the relations between membrane fluidity and rheological properties of cells.

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