DETERMINATION OF FLUORESCENCE POLARIZATION OF MEMBRANE PROBES IN INTACT ERYTHROCYTES

Possible Scattering Artifacts

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ABSTRACT The anisotropy of the fluorescence of diphenylhexatriene has been reported to be less in the membranes of intact erythrocytes than in erythrocyte ghost membranes or in membranes prepared from erythrocyte lipids. Evidence is presented that this may be an artifact due to the intense light scattering by the intact erythrocytes.

INTRODUCTION

Aloni and co-workers (1) reported that the "microviscosity" of the hydrophobic core of the plasma membrane of intact normal human erythrocytes was significantly less that than of erythrocyte ghosts. They used the depolarization of the fluorescence of the hydrophobic probe 1,6-diphenyl-1,3,5-hexatriene (DPH) to estimate the viscosity of the hydrocarbon region of the erythrocyte membrane. In another study, Aloni et al. (2) used similar techniques to estimate membrane fluidity in erythrocytes from patients with hereditary spherocytosis.

The conclusion that the membrane of the intact erythrocyte is more fluid than that of erythrocyte ghosts or of liposomes prepared from erythrocyte lipids (1) is of potential significance and suggests a fluidizing effect of erythrocyte membrane proteins that is largely lost when ghosts are formed. These findings are not consistent with the studies of Tanaka and Ohnishi (3) who used lipophilic spin labels to assess the effects of ghost formation on the hydrophobic membrane core of human erythrocytes. They found a slight asymmetry between the two halves of the lipid bilayer of the membrane with respect to fluidity. The asymmetry was lost upon ghost formation unless ghosting took place under particularly "gentle" conditions. In no case, however, was the average membrane fluidity reported (as by the spin labels) altered upon ghost formation.

The differences between the fluorescent probe and spin label studies led us to consider the possibility that the fluorescent probe results might be due partly to artifacts. The present study suggests that fluorescence depolarization of DPH in intact human erythrocytes may be significantly affected by the depolarization of exciting and emitted light due to the intense scattering of light by the erythrocytes.

Lentz et al. (4) have emphasized that when there are membranes that scatter light appreciably it is necessary to take into account the depolarization that is caused by the light scattering. Lentz et al., following the approach of Teale (5), extrapolate their values of fluorescence anisotropy to infinite dilution to obtain an anisotropy that is not influenced by scattering. Our results suggest that with the probe diphenylhexatriene in suspensions of human erythrocytes, extrapolation of the fluorescence anisotropy to infinite dilution may yield a value that is still influenced by light scattering. The anisotropy values obtained with erythrocyte ghosts and sonicated erythrocyte membrane vesicles appear to be less influenced by light scattering, but the anisotropies obtained are somewhat dependent on the method used to prepare the membranes.

METHODS

Preparation of Intact Erythrocytes, Ghosts, and Membrane Vesicles

Suspensions of intact erythrocytes, sonicated erythrocyte membrane vesicles, and osmotically produced erythrocyte ghosts were prepared from freshly drawn blood as described below, keeping constant the total concentration of hemoglobin at any given cell concentration. To make erythrocyte membrane vesicles, intact erythrocytes were bombarded with ultrasound using a 0.3-cm diameter titanium horn with the model W-375 Sonifier (Heat Systems-Ultransonics, Inc., Plainview, NY) set at 180 W for 3 min. Cells suspended in water lysed and became clear to the eye; we call this preparation erythrocyte ghosts. Samples of the preparations were analyzed for hemoglobin by the cyanmethemoglobin method (6) and for lipid phosphorus by the method of Bartlett (7).

Labeling of Erythrocytes, Erythrocyte-membrane Vesicles, and Erythrocyte Ghosts with DPH

We added 0.3 ml of DPH (1 mM in tetrahydrofuran) to 200 ml of 0.154 M NaCl and mixed thoroughly. This solution was then incubated at 37°C for 45 min with constant stirring. Erythrocytes, erythrocyte ghosts, or

erythrocyte membrane vesicles were then incubated for 20–30 min in the saline solution of DPH using various concentrations of membrane and varying the DPH concentration to keep constant the moles of DPH per mole of membrane lipid.

Measurement of Fluorescence Anisotropy

Fluorescence measurements with the labeled cells and membrane preparations were made with an Aminco Fluorometer (American Instrument Co. Silver Spring, MD) equipped with a 50 W mercury lamp. A 365-nm interference filter was used in the excitation beam and the emitted light passed through a Polacoat polarizing filter (Polacoat Division, 3M Corp., St. Paul, MN) and then through one of two film polarizers, one of which was oriented parallel to the direction of polarization of the exciting light and the other oriented perpendicular to it. The fluorescence anisotropy (r) was computed from $r = (I_\parallel - I_\perp)/(I_\parallel + 2I_\perp)$, where $I_\parallel$ is the fluorescence intensity observed parallel to the direction of polarization of the exciting light and $I_\perp$ is the fluorescence intensity observed perpendicular to it.

The apparent intensity due to light scattering by the erythrocytes and membrane preparations was a significant fraction of the total fluorescence intensity and was corrected for by subtracting $I_\parallel$ and $I_\perp$ due to scattering by a similar suspension of unlabeled membranes from $I_\parallel$ and $I_\perp$ were used to compute the anisotropy of fluorescence (8).

The highest level of DPH used gave about 1 mol of DPH per 100 mol of membrane lipid. Doubling the ratio of DPH/membrane lipid or halving the ratio caused no change in the anisotropy of fluorescence.

Preparation of Phosphatidylcholine Vesicles

Phosphatidylcholine from egg yolks (Singleton [9]) was purchased from Sigma Chemical Co. (St. Louis, MO). Vesicles were prepared by sonication of the phosphatidylcholine in an aqueous buffer (0.1 M KCl, 0.01 M Tris-HCl, pH 8) after the method of Huang (10) using the 0.3-cm diameter probe of the model W-375 Sonifier (Heat-Systems Ultransonics). The lipid phosphorus content of the vesicles was estimated by the method of Bartlett (7).

Use of Glass Beads to Create Light Scattering

We added 0.3 g of glass beads 1–5 μm diameter (Duke Scientific, Corp., Palo Alto, CA) to 10 ml 0.154 M NaCl and mixed with a vortex mixer. Aliquots of the suspension of glass beads were added to erythrocyte vesicle suspensions to determine the effect of the increased light scattering on the measured anisotropy of fluorescence of DPH. The scattering induced by the glass beads was estimated by measuring the turbidity increase caused in an erythrocyte vesicle suspension by adding various quantities of glass beads. Turbidity was measured as the absorbance at 365 nm (the exiciting wavelength).

RESULTS AND DISCUSSION

Dependence of the Fluorescence Polarization on Cell Concentration

As shown in Fig. 1 the anisotropy of the fluorescence of the DPH label depends on (a) the concentration of cells, in the case of intact erythrocytes, (b) the equivalent cell concentration for erythrocyte membrane vesicles prepared by sonication and in erythrocyte ghosts prepared by osmotic lysis. The values we obtained for the membrane vesicles and erythrocyte ghosts are similar to those obtained by others (1, 2). The anisotropies that we obtained with intact cells are considerably smaller than those with erythrocyte

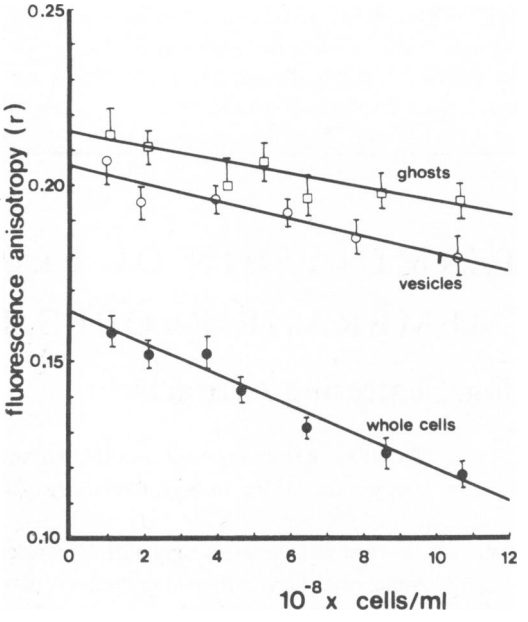

FIGURE 1 Fluorescence anisotropy of DPH in erythrocyte membranes as a function of cell concentration for intact cells (●), ghosts produced by osmotic hemolysis (□), and vesicles produced by sonication (○), all at 37°C.

membrane vesicles and erythrocyte ghosts as reported by Aloni et al. (1). The dependence of anisotropy on cell concentration is greater for the intact cells than for the membrane vesicles and ghosts. Extrapolation of the curve for the intact cells to zero cell concentration does not give an anisotropy that is consistent with the y-intercept of the curves for the membrane vesicles or erythrocyte ghosts. We were concerned that the apparently lower anisotropy of DPH fluorescence in intact erythrocytes might be due to artifact. We investigated two potential sources of artifact: the presence of a higher concentration of hemoglobin, and a greater degree of light scattering in the intact erythrocytes compared with the erythrocyte ghosts.

Effects of Addition of Hemoglobin to DPH-labeled Erythrocyte Membrane Vesicles and Phosphatidylcholine Vesicles

The fluorescence of DPH was not detectable in hemoglobin solutions. This suggests that DPH does not bind to a hydrophobic domain of hemoglobin where it fluoresces with a relatively low anisotropy. This does occur in solutions of bovine serum albumin. Various amounts of hemoglobin solution (6.2×10^{-4}M heme) were added to DPH-labeled erythrocyte vesicle suspensions and the resulting fluorescence anisotropy determined. Over the range of hemoglobin concentration of 0–40 μM heme there was no significant effect of added hemoglobin on the anisotropy of DPH fluorescence.

One possible problem with the experiments just

described is that, if the sonicated vesicles are principally right-side out, then adding hemoglobin to the vesicle suspension may not allow the same interaction between hemoglobin and DPH that occurs in intact erythrocytes. For this reason, we also investigated this problem in lecithin vesicles where membrane proteins cannot interfere. Experiments were performed with suspensions of phosphatidylcholine vesicles labeled with DPH. Addition of hemoglobin to the labeled vesicles caused no significant change in the anisotropy of DPH fluorescence in the range of heme concentration from 0 to 40 μM.

The highest concentration of hemoglobin used in these experiments is about 500 times less than that in an intact erythrocyte. Increasing the hemoglobin concentration further led to such attenuation of the fluorescence signal that the measurements could not be accurately made. Because we used relatively dilute hemoglobin solutions our data do not unequivocally rule out the possibility that hemoglobin in the intact erythrocytes is responsible for the lowered fluorescence anisotropy of DPH. However, since the high concentrations of hemoglobin used (40 μM) had no effect whatever on DPH fluorescence anisotropy, the results suggest that hemoglobin is not responsible for the observed fluorescence depolarization. 8-Hydroxy-1,3,6-pyrenetrisulfonate, which is quenched by hemoglobin, is quenched by hemoglobin concentrations as low as 4 μm (11). There is also strong evidence that DPH is buried in the hydrophobic domain of the acyl chains of membrane phospholipids (8), where it should be inaccessible to hemoglobin.

Effects of Light Scattering Induced by Adding Glass Beads

Addition of glass beads to DPH in solution. To a solution of DPH (1.5 μM) in paraffin oil we added various amounts of glass beads (1–5 μm diameter). The degree of light scatter due to the presence of the glass beads was estimated by the optical density at 365 nm (the exciting wavelength). With increasing concentration of glass beads the anisotropy of DPH fluorescence fell. The plot of anisotropy of DPH fluorescence vs. OD_{365} was linear (over the range of OD_{365} from 0 to 0.6) with a slope of -0.0452 and linear regression coefficient of -0.9976. This suggests that the glass beads can cause depolarization of DPH fluorescence in the absence of membranes.

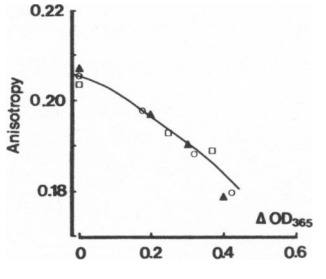

FIGURE 2 The anisotropy of DPH fluorescence in erythrocyte membrane vesicles as affected by the addition of glass beads. The DPH was first equilibrated with sonicated erythrocyte membrane vesicles at various concentrations and then aliquots of a suspension of glass beads were added. The final equivalent concentration of cells present was quantified by means of lipid phosphorus determinations to be 1.8×10^8 cells/ml (○), 2.38×10^8 cells/ml (□), and 3.48×10^8 cells/ml (▲).

Effect of Scattering Induced by Addition of Glass Beads to DPH-labeled Erythrocyte Membrane Vesicles

Addition of glass beads (1–5 μm diameter) caused an increase in the optical density of the suspension that was linear with the amount of glass beads added and also a decrease in the fluorescence anisotropy of the DPH. Fig. 2 shows the dependence of the fluorescence anisotropy on the optical density due to the glass beads at 365 nm (the exciting wavelength) for different concentrations of erythrocyte membrane vesicles. In the range of concentrations of erythrocyte membranes investigated, the effect of glass beads on the anisotropy of DPH fluorescence was independent of the concentration of membrane vesicles.

Fig. 3 compares the effects on the fluorescence anisotropy of DPH of the scatter due to intact erythrocytes with the scatter caused by adding glass beads to erythrocyte membrane vesicles. In diluting both the erythrocyte and the erythrocyte membrane vesicles the ratio of DPH to membrane lipid was kept constant. The intact erythrocytes could not be diluted to a greater extent than that shown and still maintain a workable signal-to-noise ratio. The OD_{365} due to scattering by the intact erythrocytes was calculated by subtracting the OD_{365} due to the hemoglobin present from the total OD_{365} of the suspension. The shape

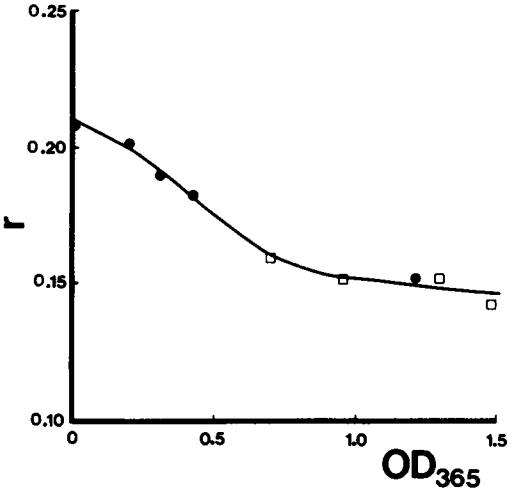

FIGURE 3 The effect of scattering on the anisotropy of DPH fluorescence for erythrocyte membrane vesicles with glass beads added to produce light scattering (●) and for intact erythrocytes (□). The optical density at 365 nm (OD_{365}) is used as an index of light scattering (see the text for an explanation of how this is computed for the intact erythrocytes).

of the curve in Fig. 3 suggests that if the erythrocytes could be diluted further, their fluorescence anisotropies would fall on a curve that would allow extrapolation to zero cell concentration to give a limiting anisotropy equal to that for the cell membrane vesicles.

In conclusion, we feel that the extrapolation procedures suggested by others (4, 5) are valid. The problem, in our opinion, is that the erythrocytes in our experiments could not be diluted sufficiently for the extrapolation methods to apply. Others working with intact erythrocytes or other highly refractile preparations may encounter similar difficulties. Our results suggest a need for caution in interpreting measurements of fluorescence polarization in the presence of highly scattering particles.

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