Biochimica et Biophysica Acta 856 (1986) 301–309
Elsevier

BBA 72997

Lateral mobility of a lipid analog in the membrane of irreversible sickle erythrocytes

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(Received November 6th, 1985)

Key words: Irreversible sickle cell; Sickle cell anemia; Fluorescence photobleaching recovery; Dioctadecylindocarbocyanine iodide; Lateral diffusion; Membrane lipid; (Erythrocyte membrane)

The major feature of sickle cell anemia is the tendency of erythrocytes to sickle when exposed to decreased oxygen tension and to unsickle when reoxygenated. Irreversible sickle cells (ISCs) are sickle erythrocytes which retain bipolar elongated shapes despite reoxygenation. ISCs are believed to owe their biophysical abnormalities to acquired membrane alterations which decrease membrane deformability. While increased membrane surface viscosity has been measured in ISCs, the lateral dynamics of membrane lipids in these cells have not heretofore been examined. We have measured the lateral diffusion of the lipid analog 3,3′-dioctadecylindocyanine iodide (DiI) in the plasma membrane of intact normal erythrocytes, reversible sickle cells (RSCs), and irreversible sickle cells by fluorescence photobleaching recovery (FPR). The diffusion coefficients ± standard errors of the mean of DiI in intact normal red blood cells (RBCs), RSCs, and ISCs at 37°C are $(8.06 \pm 0.29) \cdot 10^{-9}$ cm$^2 \cdot$ s^{-1}, $(7.74 \pm 0.22) \cdot 10^{-9}$ cm$^2 \cdot$ s^{-1}, and $(7.29 \pm 0.24) \cdot 10^{-9}$ cm$^2 \cdot$ s^{-1}, respectively. A similar decrease in the diffusion coefficient of DiI in the plasma membranes of the three cell types was observed at 4, 10, 17, 23, and 30°C. ANOVA analysis of the changes in DiI diffusion showed significant differences between the RBC and ISC membranes at all temperatures examined. The characteristic breaks in Arrhenius plots of the diffusion coefficients for the RBCs, RSCs, and ISCs occurred at 20, 19, and 18.6°C, respectively. Photobleaching recovery data were used to estimate (Boullier, J.A., Melnykovich, G. and Barisas, B.G. (1982) Biochim. Biophys. Acta 692, 278–286) the microviscosities of the plasma membranes of the three cell types at 25°C. We find significant differences between our microviscosity values and those obtained in previous fluorescence depolarization studies. However, both methods indicate qualitatively similar differences in membrane microviscosity amoung the various cell types.

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Abbreviations: ISC, irreversible sickle cell; RSC, reversible sickle cell; FPR, fluorescence photobleaching recovery; RBC, red blood cell; HbS, hemoglobin S; DPH, 1,6-diphenyl-1,3,5-hexatriene; DiI, dioctadecylindocarbocyanine iodide; E_a, activation energy.

Introduction

Sickle cell disease is the result of a genetically determined abnormality of the primary structure of the hemoglobin molecule β-chain. When deoxygenated the abnormal hemoglobin S (HbS) aggregates to form long paracrystalline arrays or tactoids which produce the typical sickle shaped erythrocyte [1–3]. Upon oxygenation the hemo-

globin reverts to the soluble state and the majority of sickle erythrocytes regain the normal biconcave structure. Such cells are referred to as reversible sickle cells (RSCs). A fraction of the sickled cells retain the abnormal morphology even when fully oxygenated [4]. These irreversible sickle cells (ISCs) constitute a relatively constant percentage of the total circulating red blood cells in any one patient. This percentage may vary from 4 to 44% [5–7]. ISCs are much less deformable than normal erythrocytes and may contribute to the initiation of microvascular occlusion common in sickle cell disease. Since the hemoglobin of oxygenated ISCs reverts to the normal soluble state it is believed that the cells' abnormal morphology is the result of an irreversible alteration of their plasma membranes [8,9].

The implied membrane lesion in ISCs has been the subject of investigation for many years. Several observations have been made which support the existence of such a membrane defect. Fragmentation and subsequent loss of plasma membrane has been observed following repeated in vitro oxygenation-deoxygenation cycles [10,11] and during metabolic depletion resulting from prolonged anaerobic incubation [12]. ISCs have been shown to be abnormally permeable to cations resulting in increased intracellular concentrations of Ca^{2+} [13–16] and decreased concentrations of K^{+} [17–19]. The decreased intracellular K^{+} concentration has been related to cellular dehydration and increased hemoglobin concentration. Moreover it has been demonstrated that there is significantly more hemoglobin bound to the membranes of ISCs than to the membranes of normal red blood cells (RBCs) of reversible sickle cells [20–23] and ISC membrane proteins have been shown to exhibit abnormal cross-linking [24]. Finally, a change in the spectrin-actin lattice of the ISC membrane has been demonstrated by Lux and co-workers [25,26].

The membranes of normal red blood cells have been extensively examined. Such studies have been facilitated by the absence in red cells of intracellular membranes, thus allowing accurate analysis of the plasma membrane composition. Several experimental approaches, including ESR spectroscopy [27–29], microaspiration techniques [30–32], fluorescence polarization [33–35], and fluorescence photobleaching recovery (FPR) [36–38], have been used to determine the membrane dynamic properties of normal erythrocytes. A recent FPR study has reported differences in the lateral mobility of phospholipids in the external and internal leaflets of erythrocyte membranes [39]. Changes in membrane lipid mobility have been related to maturation [27,29,32,33], muscular dystrophy [34], hypercholesteremia [24,37,38], and spherocytosis [39].

Although differences in static properties of ISC and RBC plasma membranes have been clearly demonstrated, few studies of ISC membrane dynamic properties have been reported. Rice-Evans et al. [40] examined the steady-state fluorescence polarization of 1,6-diphenyl-1,3,5-hexatriene (DPH) in the membranes of sickle cells and normal erythrocytes. Their results indicated that the hydrophobic region of the sickle cell membrane is less fluid than that of normal erythrocytes. The viscoelastic properties of normal erythrocytes, RSCs, and ISCs have been examined by Nash, Johnson and Meiselman [41] using micropipette aspiration techniques. They conclude that the membrane surface viscosity of the ISC is greater than two times that of normal RBCs.

With the exception of the fluorescence photobleaching recovery study of Bloom and Webb [38] and the microaspiration studies of Nash and Meiselman [32,41], all of the 'fluidity' studies cited above were performed on red blood cell membrane fragments or ghosts. It has, however, been demonstrated that red blood cell lysis damages the spectrin-actin lattice [42–44]. We report here a fluorescence photobleaching recovery study of the lateral mobility of the fluorescent lipid analog 3,3′-dioctadecylindocarbocyanine iodide (DiI) in the plasma membranes of intact normal RBCs, RSCs, and ISCs.

In view of previous work, we attempted to answer three questions. First, what is the effect of irreversible sickling on the lateral mobility of DiI? Second, what effect does temperature have on the lateral mobility of DiI in the three cell types examined? Third, how do our results performed on intact cells compare with those previously performed on lysed cells?

Material and Methods

Cell preparation. Fresh blood was collected in 10 ml Vacutainer tubes (Becton-Dickinson, Rutherford, NJ) containing an anticoagulant (acid/citrate/dextrose A solution). The choice of anticoagulant has been shown by previous researchers to have no discernable effect on the formation of ISCs [45]. Blood samples obtained from patients with clinical histories of sickle cell disease were the kind gifts of Dr. Marilyn Johnston, St. Louis University School of Medicine, St. Louis, MO. That these patients were in fact homozygous for hemoglobin S was verified by cellulose acetate strip electrophoresis in Tris-veronal buffer (pH 8.6). Control samples of normal human blood were obtained from assorted, apparently healthy volunteers by arm venipuncture. Whole blood samples were treated with 1 vol% of a solution of penicillin (10000 U/ml) and streptomycin (10000 μg/ml). The treated blood samples were divided into 1-ml aliquots and stored at 4°C. All experiments were performed within 5 days of collection of each blood sample. Cells used in these experiments were treated identically regardless of the source. Oxygenation of all samples was carried out by exposure to room air for 30 min, after which time the cells exhibited a bright red color. 10 μl of oxygenated blood were added to 0.45 ml of phosphate-buffered saline (PBS, 0.122 M NaCl, 0.03 M $KH_2PO_4 + Na_2HPO_4$, 0.2 g/dl glucose (pH 7.44), 300 mosmol/kg), labeled with DiI as described below, and used immediately for the lateral diffusion measurements. ISCs were identified as cells whose length was greater than twice the width when oxygenated, as defined by Lessin et al. [23]. Blood samples examined from individuals homozygous for HbS contained on the average approx. 20% ISCs under oxygenated conditions. Of the remaining RSCs only those cells showing a normal biconcave morphology were selected for diffusion measurements.

Cell labeling for fluorescence photobleaching recovery measurements. The phospholipid analog DiI was synthesized in accordance with the procedure of Sims and co-workers [46]. The DiI was dissolved in absolute ethanol to a working solution of 0.5 mg/ml. Each 10 μl blood sample diluted in 4.95 ml of phosphate-buffered saline was treated with 50 μl of the working DiI solution. The resultant solution containing approx. 5 μg/ml of DiI was vortexed gently and incubated 5 min at 37°C. Following incubation the cells were centrifuged at 1500 to 2000 rpm and washed with 1 ml of phosphate-buffered saline containing 3 mg/ml of bovine serum albumin (PBS/BSA solution). The washing procedure was repeated once and the cells were finally resuspended in 0.5 ml of the PBS/BSA solution. Difficulties due to crenation of the labeled cells during examination were alleviated by using siliconized well slides and plastic coverslips. The depression of the well was 3 mm in diameter by 0.08 mm deep. Fluorescence photobleaching recovery measurements were carried out within 20 min of the labeling procedure.

Preparation of DiI monolayer. A solution of DiI in diethyl ether was layered on the surface of distilled deionized water in a 50 ml beaker. The beaker was placed in an incubator at a temperature of 37°C for 1 h to evaporate the diethyl ether. U.S.P. extra heavy mineral oil was carefully layered atop the surface of the water. The resulting DiI monolayer was readily visualized at the oil/water interface using oil immersion optics and illuminated with 514.5 nm laser light to excite DiI fluorescence. Monolayers of 5% and 25% coverage of the interfacial area with DiI were prepared with 2 μg and 10 μg of the probe, respectively. The lateral diffusion of DiI in each monolayer was measured at 22°C. The mineral oil viscosity was measured at selected temperatures in a calibrated Cannon-Fenske viscometer mounted in a water bath.

Fluorescence photobleaching recovery measurements. Our current equipment and methods for FPR methods are published elsewhere [47–53]. Briefly, fluorescence from cells labeled with DiI is excited by 0.2 μW of 514.5 nm light from an argon ion laser. Upon briefly increasing the beam intensity 10^3–10^4-fold, fluorophores in the 0.83 μm ($1/e^2$ radius) illuminated spot are irreversibly photobleached. A special light pulse generator achieves these rapid, high-intensity bleaching pulses without introducing measureable beam deviations [51]. A Zeiss Universal microscope with an MP03 microscope photometer affords visual observation and photometric measurement of fluorescence via photon counting. A thermoelectri-

cally-controlled stage maintains sample temperature. An iterative, non-linear algorithm implemented in BASIC on a NOVA 3/12 computer affords direct, on-line data reduction. We obtain, among other parameters, the diffusion coefficient and the mobile fraction of fluorophores.

Two features recently introduced into our system have proved particularly useful. First, a beam splitter has been positioned so that the field under examination may be briefly viewed under fluorescence illumination without intercepting the laser beam with moving optical elements. Second, accurate focusing the laser beam while using low excitation beam intensities has been greatly facilitated by interfacing a NITEC NVS-100 image intensifier to the lateral port of the microscope.

The photobleaching of intact erythrocytes presents two problems, the absorption of the excitation light by hemoglobin and the photolability of the intact erythrocytes following bleaching pulse intensities greater than 10000 W/cm^2 [54,55]. We circumvented these difficulties by using extremely low bleaching power levels of less than 0.2 mW. We have calculated the steady-state local heating expected for bleaching an RBC with 0.2 mW of 514.5 nm light in a spot of 1 μm $1/e^2$ radius to be less than 2 K. This might be expected to be a safe bleaching power level since the lowest melting transition temperature of the RBC membrane is about 51°C. This expectation is realized experimentally since diffusion coefficients determined from multiple bleaches, three or more, on the same spot did not vary by more than $\pm 0.50 \cdot 10^{-9}$ cm$^2 \cdot$ s^{-1}. Moreover no visible membrane damage or hemolysis results from repeatedly bleaching the same cell several tens of times at this power level. The absolute accuracy of diffusion coefficients measured on lipids and lipid analogs is estimated to be about $\pm 20\%$ with precision closer to $\pm 5\%$. The diffusion coefficients reported here represent average values obtained from 15 to 38 cells.

Microviscosity of the cell membranes. This was estimated by comparing DiI diffusion coefficients measured at water/mineral oil interfaces with diffusion coefficients measured in the plasma membranes of the cells. As described earlier, the viscosity of the pure mineral oil used to form the interfaces was determined at selected temperatures. The equation

$$D \propto T/\eta \quad (1)$$

may be used to describe the relationship between viscosity and diffusion where D is the diffusion coefficient, T is the absolute temperature, and η is the viscosity of the medium in which the diffusion occurs. All three parameters may be measured for a DiI monolayer at the mineral oil/water interface. The proportionality constant in Eqn. 1 can thus be evaluated. Knowledge of this constant permits the estimation of η for cell membranes where D has been measured at a known temperature.

Results

In order to assess the effects of irreversible sickling on the lipid mobility in the membranes of erythrocytes of patients with homozygous sickle cell disease, we measured the lateral mobility of DiI at 4, 10, 17, 23, 30, and 37°C in normal erythrocytes, RSCs, and ISCs. Table I shows the results of this experiment (mean values ± S.E.). We established a diffusion coefficient for DiI in the plasma membrane of normal intact erythrocytes of $(8.06 \pm 0.29) \cdot 10^{-9}$ cm$^2 \cdot$ s^{-1}. The diffusion coefficient for DiI in the membranes of RSCs and ISCs was $(7.74 \pm 0.22) \cdot 10^{-9}$ cm$^2 \cdot$ s^{-1} and $(7.29 \pm 0.24) \cdot 10^{-9}$ cm$^2 \cdot$ s^{-1}, respectively. A similar order in DiI diffusion coefficients among the

TABLE I

DiI DIFFUSION COEFFICIENTS IN NORMAL ERYTHROCYTES, REVERSIBLE SICKLE CELLS, AND IRREVERSIBLE SICKLE CELLS

The collection and treatment of the cells are described in Materials and Methods. The diffusion coefficient D for each cell type at each temperature is presented as mean ± S.E. of n individual cell measurements, where n is the number given in parentheses following D.

Temp. (°C)	Diffusion coefficient (D) (10^{-9} cm$^2 \cdot$ s^{-1})		
	RBC	RSC	ISC
4	2.10 ± 0.13 (38)	1.93 ± 0.12 (19)	1.61 ± 0.09 (20)
10	3.06 ± 0.18 (20)	2.81 ± 0.17 (15)	2.51 ± 0.12 (15)
17	4.43 ± 0.18 (20)	4.30 ± 0.20 (18)	3.86 ± 0.14 (19)
23	5.71 ± 0.20 (19)	5.44 ± 0.17 (19)	4.96 ± 0.24 (18)
30	6.82 ± 0.25 (19)	6.38 ± 0.25 (18)	6.05 ± 0.23 (18)
37	8.06 ± 0.29 (16)	7.74 ± 0.22 (17)	7.29 ± 0.24 (17)

three cell types was observed at all temperatures examined. The difference in the diffusion coefficient of DiI in the membranes of RBCs, RSCs, and ISCs was the greatest at 4°C where values of $(2.10 \pm 0.13) \cdot 10^{-9}$ $cm^2 \cdot s^{-1}$, $(1.93 \pm 0.12) \cdot 10^{-9}$ $cm^2 \cdot s^{-1}$, and $(1.61 \pm 0.09) \cdot 10^{-9}$ $cm^2 \cdot s^{-1}$, respectively, were observed. ANOVA analysis of differences between the mean DiI diffusion constants for the three cell types was performed with Duncan's Multiple Range Test for Variability. The differences between DiI diffusion coefficients in the membranes of normal RBCs and ISCs were statistically significant at each individual temperature with a probability $P > 95\%$. The mean diffusion coefficients of the probe in the membrane of the RSCs were not significantly different from their values in the membranes of either ISCs or RSCs.

Arrhenius plots of the data are shown in Fig. 1. The clear break at 18.6°C in the plot for ISCs represents a decrease in the apparent activation energy (E_a) from approximately 11 to 5 kcal/mol. In contrast the breaks at 20°C and 19°C in the Arrhenius plots of the RBC data and RSC data, respectively, represent decreases in E_a from approximately 9.5 to 4.5 kcal/mol. The temperatures at which breaks in linearity of Arrhenius plots occur do not differ significantly among each of the three cell types. However, the activation energies and their temperature dependences for probe diffusion in the RSC and RBC membranes parallel each other rather closely and differ demonstrably from the pattern observed for the ISCs.

The per cent recovery of probe fluorescence following photobleaching represents that fraction of the probe which is mobile in the membrane. Various studies show that the mobile fraction of DiI is close to 100% in the majority of biological membranes. Table II shows the average per cent recovery of fluorescence following photobleaching DiI in the membranes of normal RBCs, RSCs, and ISCs at 4, 10, 17, 23, and 37°C. No significant difference in recovery was observed with regard to temperature or cell type. The average per cent recoveries of DiI fluorescence in the RBC, RSC, and ISC membranes averaged over all temperatures examined were 93.4 ± 6.8, 94.9 ± 5.3, and 92.2 ± 7.1, respectively. Mobility of the DiI probe was thus 100% to within experimental uncertainty in all cases observed.

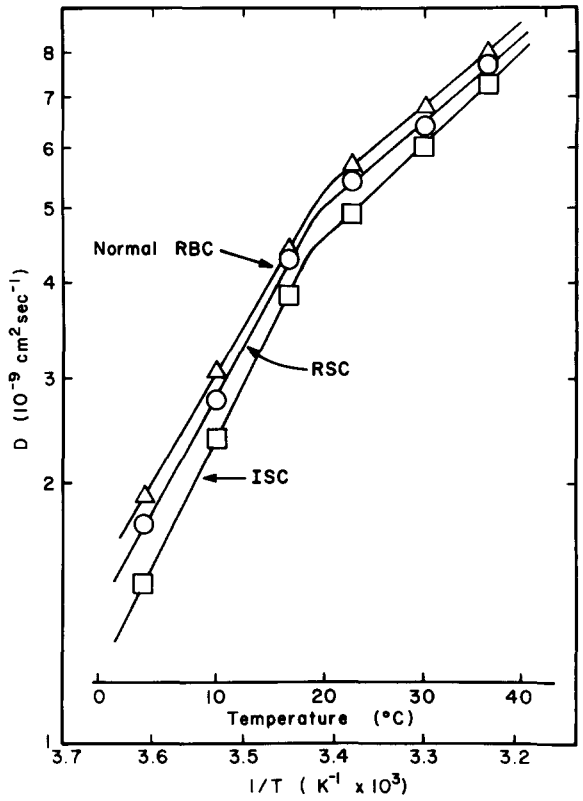

Fig. 1. Diffusion results for DiI-labeled intact normal erythrocytes (△), reversible sickle cells (○), and irreversible sickle cells (□). These Arrhenius plots represent composites for four normal donors and three donors homozygous for sickle cell anemia. Each point represents the average of approx. 20 cells per respective data point. Due to the relatively large number of cells used per point, bars representing the standard errors of the means fall within the dimensions of the symbols used to designate the points and have been omitted for clarity. The standard error of the mean for each point may be found in Table I.

Rice-Evans and co-workers [40] have previously published the results of steady-state fluorescence polarization studies of diphenylhexatriene (DPH) on normal erythrocyte ghosts and ghosts of erythrocytes from sickle cell disease patients. Two samples of erythrocyte ghosts were examined in that study. One contained a significant proportion of ISCs (Group A) and the second contained very few ISCs (Group B). These authors analyzed fluorescence polarization data for the three cell types by calculating membrane microviscosities via the Perrin equation using a method first suggested by

TABLE II

PER CENT RECOVERY OF DiI FLUORESCENCE FOLLOWING PHOTOBLEACHING IN NORMAL ERYTHROCYTES, REVERSIBLE SICKLE CELL AND IRREVERSIBLE SICKLE CELLS

The collection and treatment of the cells are described in Materials and Methods. The per cent recovery value for each cell type at each temperature is presented as mean ± S.D. of 15–38 individual cell measurements.

Temp. (°C)	Per cent recovery (mobility)		
	RBC	RSC	ISC
4	92.7 ± 9.2	97.4 ± 7.2	98.5 ± 6.6
10	95.8 ± 10.8	97.5 ± 3.7	95.9 ± 6.0
17	92.6 ± 7.5	97.0 ± 4.9	92.3 ± 6.4
23	96.1 ± 4.4	94.9 ± 4.4	90.7 ± 5.1
30	93.9 ± 5.3	93.6 ± 6.1	91.2 ± 5.8
37	94.6 ± 3.1	90.5 ± 6.7	94.1 ± 10.0

Shinitzky and co-workers [56,57]. We have previously shown how membrane microviscosities calculated from steady-state fluorescence polarization data by this equation can be compared with microviscosities calculated from fluorescence photobleaching recovery data [50]. We have calculated the membrane microviscosities of intact RBCs, RSCs, and ISCs at 25°C. The results of these calculations are presented in Table III. The values calculated from the DPH fluorescence polarization studies on ghost RBCs, RSCs, and ISCs from the studies cited above [40] are included in Table III for comparison. Comparison of the microviscosity values for normal RBCs estimated by the two techniques shows remarkable agreement (6.16 and 6.22 poise). For the sickle cells the membrane microviscosities obtained by the two techniques are quite different although changes associated in each case are qualitatively similar. Microviscosities obtained by DPH fluorescence polarization measurements were significantly larger than the values obtained via photobleaching.

TABLE III

COMPARISON OF ERYTHROCYTE MEMBRANE MICROVISCOSITIES ESTIMATED FROM DPH FLUORESCENCE POLARIZATION AND FROM FLUORESCENCE PHOTOBLEACHING RECOVERY

The collection and treatment of the cells are described in the text. The diffusion values represent the mean of multiple measurements. The viscosity values from DPH fluorescence polarization are from Rice-Evans et al. (1978) [40]. All measurements were made at 25°C.

Cell type (Boullier/ Rice-Evans)	Diffusion coefficient (10^{-9} cm^2·s^{-1})	Viscosity (poise)	
		from D	from fluorescence polarization of DPH
RBC/Normal	6.05 ± 0.78	6.16	6.22 ± 0.23
RSC/SS Group B	5.73 ± 0.67	6.50	8.10 ± 0.44
ISC/SS Group A	5.29 ± 0.92	7.04	10.63 ± 0.33

Discussion

We have measured the lateral diffusion of the fluorescent lipid analogue DiI in the membranes of intact normal erythrocytes, RSCs, and ISCs. The probe was highly mobile at all temperatures measured in a range from 4 to 37°C. The absolute values of our diffusion constants for intact normal erythrocytes are in good agreement with those reported by Bloom and Webb [38] for temperatures below 20°C. At temperatures above 20°C, however, our values are considerably lower than those reported by Bloom and Webb [39] until at 37°C they differ by a factor of more than two. The difference might be attributed to the fact that Bloom and Webb used a different cyanine dye, 3,3′-dioctadecylindodicarbocyanine iodide (DiI-C_{18}-[5]), as their probe. This is a somewhat spacially-extended molecule in comparison to DiI and may seek a different microenvironment at temperatures above 20°C. Nevertheless, comparison of our diffusion coefficients with those previously reported for DiI fluorescence photobleaching recovery studies on erythrocyte ghosts supports the assertion that the membrane properties of the intact cells differ significantly from ghosts [38,58]. Our DiI diffusion coefficients are approximately 2–4-times greater than those previously reported for ghosts [36,37]. Bloom and Webb [38] have extensively discussed possible explanations for these differences.

A characteristic break in the Arrhenius plots of the DiI diffusion coefficients was observed in all three cell systems. Such breaks may be interpreted as representative of a membrane structural change at the temperature at which the break is observed. We observe this change at 20°C in normal intact

erythrocytes and this result is in good agreement with previous observations using several different techniques [58–61]. In previous photobleaching studies, such breaks in linearity have been reported at somewhat lower temperatures. In the photobleaching study on intact erythrocytes [38] a break at 12°C was noted, while photobleaching measurements on erythrocyte ghosts indicate an inflection between 12 and 17°C [36]. The apparent activation energy (E_a) calculated from our diffusion data is approx. 9.5 kcal/mol below 20°C and 4.5 kcal/mol above 20°C. Bloom and Webb [38] observed a similar change of E_a at 12°C from approx. 25 to 14 kcal/mol. In contrast, Cooper et al. [34] estimated human RBC ghost membrane microviscosity via DPH fluorescence polarization from 10 to 40°C. These workers determined E_a to be 8.3 kcal/mol but did not observe a break in the slope of the Arrhenius plot.

Membrane microviscosities calculated from our lateral diffusion studies are in fair agreement with those previously calculated from DPH fluorescence polarization data via the Perrin equation [40]. Various questions have been raised about the significance of quantitative membrane microviscosities estimated by this latter method. Studies have shown that DPH is not confined to the membrane in intact cells [62], that DPH rotations measured in lipid vesicles exhibit hindered tortional motions [63], and that DPH fluorescence does not decay as a single exponential [64,65]. Analysis of time-resolved fluorescence anisotropy [66] have led to the conclusion that steady-state polarization data are best evaluated by an order parameter reflecting the degree of lipid orientation within the membrane [67,69]. Nevertheless, it appears to be widely believed that membrane microviscosities calculated from steady-state polarization data provide a qualitatively correct indication of changes in membrane ordering. Although the effect of irreversible sickling on membrane microviscosity is less pronounced when inferred from DiI lateral diffusion than when estimated from DPH fluorescence polarization, the results yielded by both methods are qualitatively similar in each case. It should be noted that photobleaching studies utilizing ghosts have shown decreased DiI diffusibility in comparison to results from intact cells. This could account for higher membrane microviscosity values being reported by Rice-Evans et al. [40] for ISC ghosts when compared to the intact ISC results we present here.

As have other studies designed to assess the effect of irreversible sickling on membrane dynamics [40,41], our results suggest that the sickling process decreases the 'fluidity' of the red cell membrane. However, our work also shows that, when membrane lipid dynamics are carefully measured in intact erythrocytes, irreversible sickling is seen to cause much smaller membrane changes than were inferred from previous studies on ghosts [40]. Accordingly, intrinsic changes in the ISC membrane bilayer may be less important than previously thought in decreasing cellular deformability. Our results therefore draw increased attention to non-lipid factors in the etiology of the ISC membrane lesion. In any case the cause of the increased membrane viscosity we observe in ISC remains unclear. Previous research has demonstrated that changes in the cholesterol to phospholipid ratio within the membrane of intact cells can change the fluidity of the membrane [34,37,38,44,72]. Since this ratio is essentially unaffected by the sickling process [40,73], it is necessary to consider alternative mechanisms for th edecrease in lipid diffusibility observed to follow the sickling process. It is possible that, as has been implied by the work of Chiu et al. [74], a rearrangment of the membrane phospholipids occurring during the sickling process could account for the decreased DiI mobility. A second possibility is that distribution of fluid lipid regions and rigid lipid-protein assemblies within the membranes of red blood cells may affect probe mobility in the membrane [36]. Others have implied that lipid-protein interactions do not substantially restrict the diffusion of lipids over the erythrocyte surface [38]. It is, however, difficult to discount the possibility that altered lipid-protein interactions affect lipid dynamics in the ISC membrane, since evidence supports increased hemoglobin-membrane interaction [20–23] and an altered spectrin-actin lattice in these cells [25,26]. Rimon et al. [39] have recently asserted that changes in the spectrin-actin lattice in spherocytosis lead to changes in the lipid mobility in the internal leaflet of the RBC membrane. A similar phenomena might be involved in decreased DiI diffusibility in ISCs. Final resolu-

tion of this question must nevertheless await further experimental results.

Acknowledgement

This work was supported in part by Basil O'Connor Grant 5-135 from the March of Dimes Birth Defects Foundation and by NIH Research Career Development Award AI-00291 to B.G.B.

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