

Percentage of Reversibly and Irreversibly Sickled Cells Are Altered by the Method of Blood Drawing and Storage Conditions

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ABSTRACT: We previously reported that the percentage of reversibly and irreversibly sickled cells (RSC and ISC, respectively) in the blood of patients with sickle cell disease is strongly influenced by the method of blood drawing (*PNAS* 91:12589, 1994). We now document the effect of blood storage conditions on the percentage of RSC and ISC. The percentage of RSC was lowest when blood was stored at 0°C, while the percentage of RSC was highest in specimens kept at 37°C. At room temperature, the percentage of RSC increased slightly over 8 hours. The percentage of ISC was also temperature dependent and was reduced significantly upon cooling. Our results showed that many ISC reverted to a discoidal shape after 3 hrs of cooling after treatment of blood with oxygen or carbon monoxide. Since no Hb S polymers were detected in ISC treated with oxygen or carbon monoxide, the time required for shape restoration may be attributed to the membrane. We measured ISC levels of 10 patients with consideration of storage temperature and compared the values with those determined by the conventional method and also with those published previously.

Keywords: sickle cell disease, reversibly sickled cells, irreversibly sickled cells, cell morphology, image analysis

INTRODUCTION

Most clinical manifestations, patho-physiologic changes, and even biochemical and biophysical changes in red blood cell (RBC) membranes in patients with sickle cell disease (SCD) can be attributed to sickling of erythrocytes due to intracellular polymerization of deoxygenated sickle hemoglobin (Hb S) (1-4). However, attempts to correlate clinical course with morphologic properties of sickle erythrocytes (SS cells) have not been successful. Common methods of blood collection and storage may not allow an accurate determination of the number of sickled cells in individual patients under study. Usually blood is collected into a vacutainer and kept for variable time periods at various temperature conditions, such as

on ice, at room temperature or even in clothing pockets where the temperature may exceed room temperature. During morphologic studies of SS cells, we noticed that both the percentage and the morphologic pattern of sickled cells from the same samples varied greatly depending on storage duration and temperature.

Although the degree of sickling is affected by temperature (5-8), systematic studies regarding morphologic changes in SS cells during blood storage at different temperatures have not been done. Rampling (9) studied the effect of CO₂ and temperature on the sickling phenomenon and found that the sickling rate could be progressively inhibited by lowering temperature from 37°C to 0°C. Mackie and Hochmuth (10) studied the influence of temperature on the rheologic proper-

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ties of sickle erythrocytes and found that the rigidity of SS cells was greater at 37°C than at 25°C when the partial pressure of oxygen was changed from 156 to 40 mm Hg. Our current study investigates the effect of temperature on the morphology of SS cells in blood drawn either into ordinary vacutainers or into vacutainers previously equilibrated with a gas mixture that mimics venous oxygen tension. We find that storage temperature affects not only the percentage of RSC and ISC but also the morphologic pattern of sickled cells. The wide variations in RSC and ISC values reported in the literature may be attributed to differences in storage conditions as well as in methods of blood drawing. Standardized collection methods and storage conditions for SS blood may be critical to obtaining consistent and reliable results for correlation of SS cell morphology with clinical course.

METHODS

Blood Collection

Blood was collected from the antecubital vein of 17 pediatric patients with homozygous SCD who were followed regularly in the Comprehensive Sickle Cell Center at The Children's Hospital of Philadelphia. At the time of blood collection none of the patients showed signs of painful episodes nor had received any blood transfusions within the previous month. To minimize sickling during blood drawing (11,12), blood was drawn after release of the tourniquet. Morphologic studies require only small volumes of blood, and the blood collected for routine clinical tests was used for these experiments. Seven blood specimens were used for time course experiments, and the remaining 10 samples were used for the determination of RSC and ISC. Time course experiments were done by dividing each sample into three tubes, previously equilibrated with 5% oxygen, and incubating them at 0°C, room temperature (22–23°C), and 37°C. Aliquots (10 µl) of blood

were removed from each tube at 2, 4, 6, 8 and 24 hours with a syringe containing two volumes of 2% glutaraldehyde solution, which was equilibrated with 5% oxygen. The air space of the syringe was also equilibrated with 5% oxygen. Due to the heterogeneity among patients, morphological data for patients were individually analyzed as advised by Serjeant et al. (13). For the determination of ISC, 10 blood specimens were treated with oxygen or carbon monoxide gas for 10 min and incubated either at room temperature or 0°C for 2–3 hrs before fixing.

Morphologic Analysis

Morphologic studies of SS cells were carried out using a computer-assisted image analysis system. This system determines the percentage of SS cells with different morphologies by measuring area, perimeter, and the short axis/ long axis ratio of each cell (14). Before determination, fixed SS cells were stained with erythrosin B as described by Horiuchi et al. (15). The stained cells were introduced into a microslide (Vitro Dynamics, Rockaway, NJ) and observed directly under a Nikon microscope (16). The image acquired from a microscope was changed to a binary (black and white) image (see Figure 1) and was analyzed by an automatic image analysis system (VIDAS version 1.3, Zeiss, Thornwood, N.Y.). More than 300 cells in each aliquot were measured for calculation of shape factors. Two shape factors, circular shape factor (CSF) and elliptical shape factor (ESF), were determined by the following equations (14).

$$\begin{aligned} \text{CSF} &= 4\pi \times (\text{area})/(\text{perimeter})^2 \\ \text{ESF} &= \text{short axis}/ \text{long axis} \end{aligned}$$

The CSF represents deviation from a circle and the ESF expresses extent of elongation. If a cell is perfectly circular, both CSF and ESF values are 1.0. The lower the CSF or ESF value, the greater the degree of deviation from a perfect circle. Microscopy alone does not reveal the heterogeneity of deformed cell shapes. However, the image

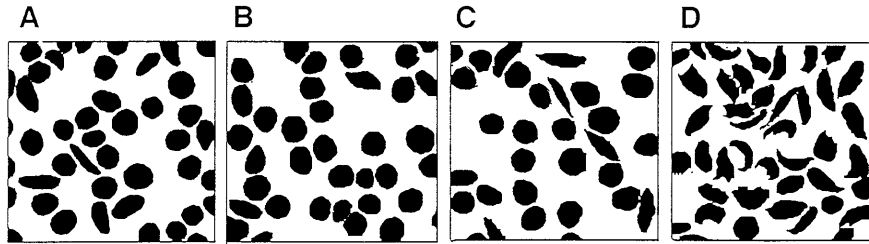


Figure 1. Effect of temperature on the morphology of SS cells in EDTA vacutainers. Blood samples, obtained from SS patients in EDTA-vacutainers or obtained under venous oxygen pressure (A) were incubated at 0°C (B), room temperature (22-23°C) (C) and 37°C (D) for 8-24 hours. Aliquots were collected and fixed with 2% glutaraldehyde solution under 5% oxygen. Suspensions of fixed SS cells were introduced into microslides, and photographed with a Mitsubishi 100U video printer connected to a Nikon microscope. The images were converted to black-and-white binary image and analyzed as described in the text. Note the formation of sickled cells at 37°C (D), while the number deformed cells is reduced with storage on ice (B).

analysis system allows rapid and accurate measurements of shape factors for many cells, and depicts the morphologic pattern as a CSF-ESF scattergraph with each point corresponding to a single cell. RSC and ISC with ESF values < 0.5 are considered elongated deformed cells (see Figure 1). For the analysis of RSC and ISC, cells with CSF values < 0.8 and ESF values > 0.5 are designated non-elongated deformed cells and include star-shaped and maple leaf or holly leaf cells. Cells with CSF values > 0.8 and ESF values > 0.5 are considered to be normal discoidal cells.

Cells treated with oxygen or carbon monoxide and having ESF values lower than 0.5 correspond to the classic definition of ISC (17-20). The percentage of ISC was determined after treatment of RBC suspension with either 100% oxygen or carbon monoxide for at least 5 min. The samples were then incubated for three hours either at room temperature or 0°C before fixing. The percentages of ISC were compared with those published previously.

Micrographs of SS Cells

Micrographs of SS cells were taken using a Nikon microscope connected to a Mitsubishi color video copy processor. As reported previously (11), if venous blood is collected and fixed with 2% glutaraldehyde solution under venous oxygen pressure, partially oxygenated sickled cells (POSC),

which have a shape similar to either ISC or raisin-like cells, are observed. However, most of these cells are RSC and are characterized by observation of dull edges. If, however, oxygenated SS cells are deoxygenated to venous oxygen pressure, sickled cells with sharp edges are formed and are described as partially deoxygenated sickled cells (PDSC). The image analysis system cannot distinguish morphological differences between POSC and PDSC. Therefore, the percentage of these cells was calculated by counting deformed cells with and without sharp edges. More than 500 cells were examined. Electron microscopy was performed with a Philips 300 electron microscope as described elsewhere (11).

RESULTS

Effect of Temperature on SS Cell Morphology

Figure 1A shows morphologic variations of a representative sample of SS cells collected in an EDTA vacutainer under venous oxygen pressure and kept for 8 hours at 0°C (Figure 1B), room temperature (Figure 1C) or 37°C (Figure 1D). As reported previously (11) and shown in Figure 1A, venous blood collected under venous oxygen pressure and fixed immediately contained POSC, discoidal cells, and a few sickled cells with sharp edges (PDSC). At 0°C, the number of elongated

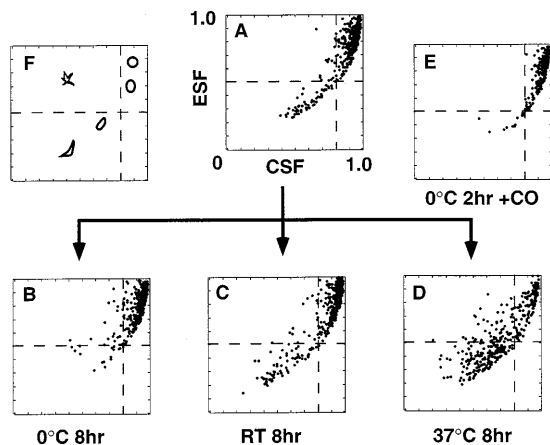


Figure 2. CSF-ESF scattergraph of SS cells stored at 0°C, room temperature and 37°C. SS cell suspensions shown in Figure 2 were analyzed by a Vidas 1.2 image analysis system. Each point in Figure 2A-2E corresponds to a cell. Note that the number of points in the bottom-left area (elongated cells) decrease drastically at 0°C with (Figure 3E) and without carbon monoxide (Figure 3B). At 37°C, points are distributed in the top-left area (star-shaped cells) and bottom-left area (elongated cells).

and non-elongated deformed cells decreased, probably due to dissociation of Hb S polymers to monomers at low temperatures (Figure 1B). Deformed cells that remain can be considered to be ISC. At room temperature, the number of elongated and non-elongated deformed cells increased slightly (Figure 1C). Upon incubation at 37°C, the number of sickled cells increased greatly (Figure 1D). Sickled cells formed during storage at 37°C are characterized by having sharp edges and are classified as PDSC.

As shown in Figure 2A, the CSF/ESF values for SS cells shown in Figure 1, exist along a diagonal line, indicating predominantly normal and elongated deformed cells. The absence of points in the top left area corresponds to the absence of non-elongated deformed cells in this blood specimen. Upon incubation at 0°C for 8 hours, the number of elongated cells decreased, as seen by the reduced number of points in the bottom left area (Figure 2B). With incubation at room temperature, the number of elongated cells increased slightly over 8 hrs (Figure 2C). At 37°C, both elongated and non-elongated deformed cells

are more numerous as seen by an increased number of points in the lower and upper left areas, respectively (Figure 2D). It should be noted that the number of deformed cells becomes negligible when SS cell suspensions are cooled to 0°C and treated with carbon monoxide (Figure 2E). Under these conditions, Hb S polymers dissociate, and by definition RSC should return to a discoidal shape. The remaining deformed cells are therefore ISC.

The percentages of elongated and non-elongated deformed cells in a sample examined at three different temperatures (0°C, room temperature and 37°C) over 8 hours are shown in Figure 3A and 3B. At 0°C, the percentage of elongated cells decreased to a minimum level (6.1%) within

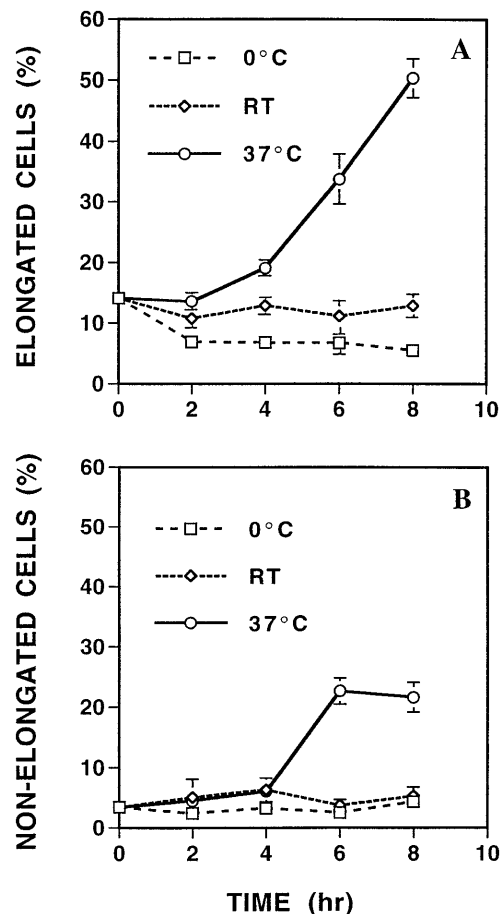


Figure 3. Effects of temperature on the percentage of elongated and non-elongated deformed cells. Percentages of elongated (A) and non-elongated sickled cells (B) were determined by an image analysis system.

Table 1. Effect of Temperature on the Percentage of Elongated Sickled Cells of 7 Patients. Blood was Collected in Either Heparin or EDTA Vacutainers under Routine Conditions or 5% Oxygen/95% Nitrogen

Pt #	Collection Tube	Temp.	% Elongated Cells					WBC	% Retic	
			0 hr	2 hr	4 hr	6 hr	24 hr			
1	Heparin	—	0°C	21.4	23.6	23.6	19.5	—	10,600	9.9
			RT	21.4	25.6	23.7	23.7	31.9		
			37°C	21.4	29.7	42.6	50.4	60.0		
2	EDTA	5% O ₂	0°C	26.6	23.9	18.7	14.7	13.7	14,200	18.4
			RT	26.6	27.0	29.6	26.2	31.4		
3	EDTA	5% O ₂	0°C	27.9	22.6	18.8	17.5	10.7	12,100	14.8
			37°C	27.9	37.5	57.4	66.0	70.2		
4*	EDTA	5% O ₂	0°C	14.2	6.1	6.9	6.3	4.8*	—	—
			RT	14.2	11.7	12.1	13.8	16.6*		
			37°C	14.2	14.3	19.9	38.4	47.5*		
5	EDTA	—	0°	19.1	15.6	9.1	11.2	6.8	12,900	12.2
			RT	19.1	16.4	—	18.8	24.7		
			37°C	19.1	43.8	58.7	59.0	60.1		
6	EDTA	—	0°C	10.2	9.0	8.1	3.5	3.8	24,600	10.7
			RT	10.2	11.6	13.0	16.9	37.7		
			37°C	10.2	31.3	43.5	44.9	50.1		
7	EDTA	—	0°C	12.2	9.9	4.3	1.3	1.3	10,700	13.9
			RT	12.2	10.4	14.4	14.9	31.3		
			37°C	12.2	16.4	27.4	41.9	68.4		

* This blood sample was incubated for 8 hours.

2 hours and remained virtually unchanged for 8 hours. The percentage of non-elongated deformed cells did not change significantly at 0°C over time (Figure 3B).

At room temperature, the percentage of elongated cells increased slightly over 8 hours incubation in all six experiments (Figure 3A). At 37°C, the percentage of elongated deformed cells increased to almost 50% after 8 hours (Figure 3A). The percentage of non-elongated deformed cells also increased during incubation at 37°C (Figure 3B).

Table 1 shows results of studies on 7 patients, including the patient described above. Although

the percentages of deformed cells at the starting points differ greatly among patients, the effect of temperature on cell sickling shows a similar trend in that percentages of elongated cells increased at 37°C and decreased at 0°C. Increased deformation over time can be attributed to sickling due to the hypoxia induced by oxygen consumption by nucleated cells. Both white blood cell and reticulocyte counts for each patient were elevated (Table 1). We found that elongated and non-elongated deformed cells formed at 37°C were mainly those with pointy edges (PDSC) as shown in Figure 4.

During the experiments shown above, we noticed that the number of ISC decreased when

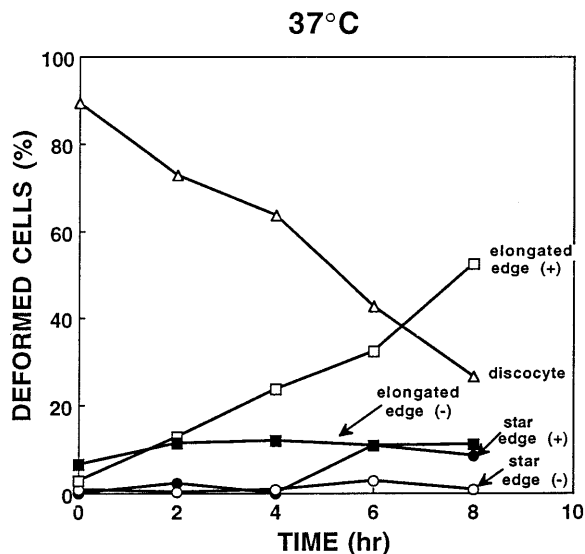


Figure 4. Effect of temperature on deformed cells with or without sharp edges during incubation at 37°C. Experimental conditions are the same as those shown in Figure 1. The number of deformed cells with sharp and cells with dull edges were calculated visually on micrographs of at least five fields and more than 200 cells. Cells with sharp edges are shown by (+), while cells with dull edges are shown by (-).

blood samples were kept on ice. To confirm this observation, we examined the percentage of ISC in SS blood incubated at 0°C, room temperature and 37°C. In these experiments, blood samples were incubated at three different temperatures for 0, 2, 4, 6, and 8 hours indicated and exposed to carbon monoxide after each incubation. As shown in Figure 5A, the percentage of elongated cells differs depending on whether blood was kept at 0°, room temperature, or 37°C. The percentages of elongated ISC measured after incubation on ice showed the lowest values, followed by those kept at room temperature and 37°C. It should be noted that the number of elongated ISC was halved after incubation at 0°C for 2 hours or longer. These results indicate the existence of ISC which do not convert to discocytes after exposure to carbon monoxide but do so after cooling on ice. There was no significant hemolysis during the incubation. Similar analyses were done for non-elongated cells. It is interesting to point out that there

3.2 ± 1.5% of irreversibly are non-elongated deformed cells (Figure 5B.). The percentage of non-elongated ISC decreased upon incubation at 0°C but did not change significantly during incubation at room temperature. The number of non-elongated ISC, however, increased from 3.2 ± 1.5% to 8.9 ± 2.5% after incubation for 8 hours at 37°C.

Determination of ISC in Clinical Blood Samples

To study the effect of storage temperature on the formation of elongated ISC of clinical samples, we treated blood with oxygen or carbon monoxide and kept it at room temperature or 0°C for 3 hours before fixing. ISC counts were much higher for blood kept at room temperature than those for blood kept at 0°C (Table 2). These results indicate that treatment of blood with oxygen or carbon monoxide alone does not convert all reversibly deformed cells to discocytes. Electron micrographs of blood samples treated with oxygen or carbon monoxide showed complete absence of Hb S fibers in 500 cells observed, indicating that Hb S in these cells is fully liganded. These results indicate that the deformation of RSC, which maintain an elongated shape after treatment with oxygen or carbon monoxide but become discoidal upon cooling, is not due to intracellular Hb S polymers.

DISCUSSION

Our current results demonstrate marked temperature-dependent differences in RBC morphology and percentage of RSC and ISC. Incubation at 37°C resulted in an increase in both elongated and non-elongated deformed cells over time. While incubation at 0°C resulted in a drastic decrease in deformed cells. The explanation of morphologic changes after incubation of blood at 0°C or 37°C are straightforward. At 0°C, deoxyHb S polymers dissociate into monomers, while at 37°C Hb S

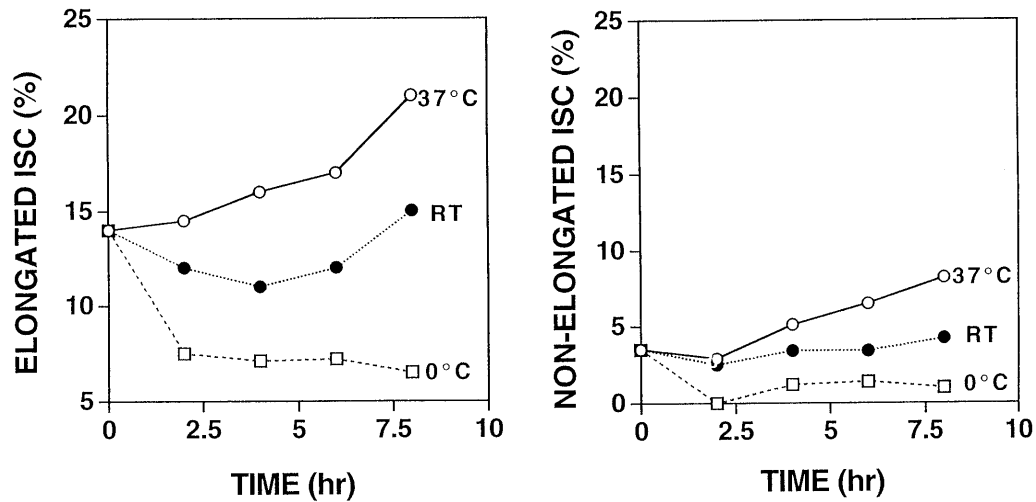


Figure 5. Effect of temperature on the percentage of elongated and non-elongated ISC. ISC were determined after treatment of SS suspensions with 100% carbon monoxide for at least 10 minutes at room temperature. Elongated ISC are cells in which the long axis is greater than twice the short axis (ESF values < 0.5). Non-elongated ISC are cells with CSF values less than 0.8 and ESF values higher than 0.5.

polymerization and cell sickling take place due to hypoxia caused by oxygen consumption by nucleated cells. All patients examined had elevated white blood cell and reticulocyte counts (Table 1). In one experiment, we incubated blood drawn in

an ordinary vacutainer at 37°C and measured the PO₂ of the blood over time. The PO₂ dropped from about 40 mm Hg to 7 mm Hg after 8 hours of incubation at 37°C. Previous reports show that cells undergo complete sickling below 20 mm Hg

Table 2. Comparison of ISC Counts Reported by Various Researchers With Our Results

Researchers	No. of Patients	Treatment	Storage	ISC (%)	Ref.
Jensen et al. (1960)	12	—	Ice	3.5 ± 3.4	24
Bertles and Milner (1968)	16	95% O ₂ :5% CO ₂	RT	28.1 ± 10.0	18
Serjeant et al. (1969)	26	95% O ₂ :5% CO ₂	RT	15.8 ± 8.2	19
Chien et al. (1970)	5	95% O ₂ :5% CO ₂	RT	20.8 ± 3.4	28
Jensen et al. (1973)	8	95% O ₂ :5% CO ₂	RT	17.8 ± 8.9	20
Rodgers et al. (1985)	5	O ₂	Ice	9.9 ± 3.2	25
	10	CO	Ice	6.5 ± 3.5	
Zipursky et al. (1993)		95% O ₂ :5% CO ₂	RT	18.1 ± 8.5	26
		CO	RT	16.2 ± 8.9	
Asakura et al. (1996)	10	O ₂	RT	12.4 ± 3.4	this paper
	10	CO	RT	9.2 ± 2.1	
	10	O ₂	Ice	5.6 ± 2.6	
	10	CO	Ice	4.1 ± 1.9	

(8,21,22) and that most sickled cells formed below 20 mm Hg possess sharp edges (11).

Our current results also show the existence of elongated ISC which convert to discocytes only after exposure to CO and incubation at 0°C. As shown in Figure 5A, the percentage of elongated cells after treatment with carbon monoxide depends on the temperature during the storage. The ISC count is higher when blood is stored at 37°C, while it is lower when blood is stored on ice. This difference is not the result of incomplete oxygenation because electron microscopy studies of more than 500 cells showed that none of the oxygenated cells contained Hb S fibers. This result suggests that some reversibly deformed cells do not change shape immediately after oxygenation of intracellular Hb S. It took 2-3 hours before ISC-like cells were restored to a discoidal shape, suggesting that the slow shape change after complete oxygenation is due to the membrane rather than Hb S polymerization. Consistent ISC values were obtained after incubation of oxygenated cells on ice for more than 3 hours.

Our current results also show the existence of non-elongated ISC. The presence of non-elongated ISC is not surprising, assuming that such cells are formed from star-shaped RSC. The presence of non-elongated cells in the densest fraction of SS blood (23) supports the existence of non-elongated ISC. Table 2 shows a comparison of our results with ISC percentages reported in the literature. Previous studies report ISC counts that are much higher than those reported by Jensen et al. (24), Rodgers et al. (25) and ourselves. Rodgers reported that there is a significant reduction in the average number of sickled forms in the peripheral blood of SCD after treatment with CO ($6.5 \pm 3.5\%$) when compared to conventional methods for ISC preparations ($14.7 \pm 8.2\%$). In contrast, Zipursky, et al. (26) reported ISC counts of $18.1 \pm 8.5\%$ and $16.2 \pm 8.9\%$ after treatment of blood with O₂ and CO, respectively. This discrepancy may be explained by differences in storage conditions after blood collection. Rodgers et al. kept blood samples on ice until the fully mixed blood was ready to be processed and then incubated at 37°C before experiments (personal com-

munication), while Zipursky et al. (26) kept blood samples at room temperature. Our results show that ISC values are affected by both ligand state and storage temperature. The percentage of ISC in blood kept at 0°C is much lower than that in blood kept at room temperature. Our unpublished results show that deformed cells that revert to discocytes at 0°C do not become elongated cells even after blood was re-warmed to 37°C. Jensen et al. (24) reported ISC counts as low as 1% (1-9%), but did not detail the method of ISC determination other than to say that samples were refrigerated prior to ISC measurements. Extremely low ISC reported in this paper may be attributed to cooling of the blood samples. Our results suggest that treatment of blood with CO followed by a 2-3 hour incubation at 0°C is the most accurate way to determine the ISC values. Wide variations in the percentage of ISC reported (18,20,24-28) may be related to differences in blood drawing and storage conditions.

As suggested by Rodgers (25) and from the results shown in this paper, fully liganded and cooled blood should be used to quantify ISC for pathophysiological studies of SCD, especially since ISC affect rheology differently than RSC. Therefore, experiments dealing with deformability, rigidity and flexibility of SS cells will be affected by the blood storage condition. Further, for experiments with oxygenated SS cells, storage temperature may alter the ISC count and affect the results. Experiments on ion transport of SS cells will also be affected by cell sickling or desickling during the storage period. Tosteson (29) reported changes in Na⁺ and K⁺ content in SS cells by sickling. Mohandas et al. (30) reported deoxygenation-induced cation permeability changes. We and others demonstrated that dehydration of SS cells is sickling dependent (23,31,32). Although little attention has been given to methods of blood drawing and subsequent blood storage, morphologic changes during storage may affect some sickling-dependent changes because sickling affects membranes, ion transport and/or metabolism of red blood cells. We recommend that for red cell pathophysiologic experiments, blood in either a

EDTA or heparin vacutainer with a rubber stopper be kept at room temperature and used within a few hours. For experiments to be done under oxygenated conditions, blood should be oxygenated immediately to prevent sickling during storage.

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