

The Sickle-Unsickle Cycle: A Cause of Cell Fragmentation Leading to Permanently Deformed Cells

By F. Padilla, P. A. Bromberg, and W. N. Jensen

We are reporting in vitro observations of sickle-unsickle transformation of freely suspended single cells (HbSS) induced by oxygenation-deoxygenation. The events were recorded by cinematography (phase optics, 16 mm cinematography). In addition, cells were fixed after progressive increments of oxygenation for subsequent scanning-beam electron microscopy. These studies seem to warrant the following conclusions regarding HbSS erythrocytes: (1) There is variability in the propensity to sickling of cells seemingly subjected to the same environment. (2) Repetitive sickling

of a single cell does not result in identical sickle deformities. (3) Disk to sickle to disk transformation may occur without apparent membrane loss or distortion or may be accompanied by the shedding of microspherules or by the loss of microspherules and permanent cell deformation. (4) Red cell "flicker" normally seen by phase-contrast cinemicrophotography disappears early in the sickling process and recurs during unsickling. (5) Sickling and unsickling as visually detected required 10–15 sec.

THE SICKLING PHENOMENON is caused by the intracellular polymerization of deoxygenated sickle hemoglobin (HbS).^{1,2} The unique distortion of the cell leads to vascular stasis, occlusion, and thrombosis.^{3,5} Intravascular hemolysis has been postulated to result from the avulsion of the rigid cellular processes as a result of the buffeting of the mechanically fragile sickled cells in the circulation.⁶ As red cells circulate, they may undergo sickling or unsickling depending on a variety of factors, such as the availability of oxygen in a particular organ at a given time, the time of exposure of the cell to a deoxygenated environment, and the duration of the sickling-unsickling process itself. The studies of Allison⁷ and Ponder⁸ suggest that the period of deoxygenation required for sickling varies between 1–4 min. Messer and Harris⁹ found viscosity changes (as indication of sickling) within 0.12 sec of sudden deoxygenation for cells from patients with homozygous sickle cell disease. To our knowledge, the duration of the in vivo sickling-unsickling process itself has not been precisely measured.

Damage to a process of a sickled cell by a focused ruby laser beam was observed by two of the authors⁸ to cause unsickling of the cell followed by

From the Department of Medicine, Division of Hematology, Little Rock Veterans Administration Hospital, Little Rock, Ark. 72206; the Department of Medicine, Division of Pulmonary Diseases, Ohio State University, Columbus, Ohio 43210; and the Department of Medicine, The George Washington University Medical Center, Washington, D.C. 20037.

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F. Padilla, M.D.: Chief, Hematology Division, Department of Medicine, Little Rock Veterans Administration Hospital, Little Rock, Ark. 72206. P. A. Bromberg, M.D.: Chief, Division of Pulmonary Diseases, Department of Medicine, Ohio State University, Columbus, Ohio 43210. W. N. Jensen, M.D.: Professor and Chairman, Department of Medicine, The George Washington University Medical Center, Washington, D.C. 20037.

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conversion of other cell processes into microspherules. These observations suggest that fragmentation of cells during the unsickling process might also occur under more physiologic conditions.

This report describes the results of experiments in which single, isolated, plasma-suspended erythrocytes from patients with homozygous sickle cell disease were repetitively sickled and unsickled by exposure to suitable gas mixtures.

MATERIALS AND METHODS

Erythrocytes from patients with homozygous sickle cell disease were suspended in a mixture of native plasma and sterile, Krebs-Ringer-phosphate buffer (pH 7.34). A drop of the cell suspension was introduced into a Prior chamber with a hypodermic needle with the bevel turned upward, so that the fluid from it first strikes the central area on the upper cover slip. The drop then falls to make contact with the lower cover slip and provides a column of plasma-suspended cells between two cover slips. The gas in the chamber surrounding the hanging column of suspended cells was humidified and was introduced through polyethylene tubes from tanks that contained 100% nitrogen or 100% oxygen. Gas flow was monitored before and during each experiment. Temperature was measured with thermal needle probes located within the hanging column in the chamber and near the cell under observation. The chamber assembly was placed in a temperature-controlled (37°C) microscope stage, and the cells were observed under phase-contrast microscopy in a Wild M-40 inverted microscope. Observations were recorded on 16 mm Kodak Plus X reversal-type film at 12 frames/sec, using a Bolex camera and a Sage cinemicrophotographic attachment. From the movie, frames were selected and prints were prepared from photographic enlargements of original negatives.

The intracellular localization and distribution of hemoglobin was also studied under Soret band microscopy. This was accomplished by placing an interference filter (specific absorption 414 nm) between the light source and the microscope condenser. Under these conditions, structures containing hemoglobin are highly absorbent and appear black.¹⁰

The experimental design allowed observation and photography of rapid changes in single cells during repetitive oxygenation or deoxygenation of freely suspended erythro-



Fig. 1. Montage of 12 frames from film sequence A.

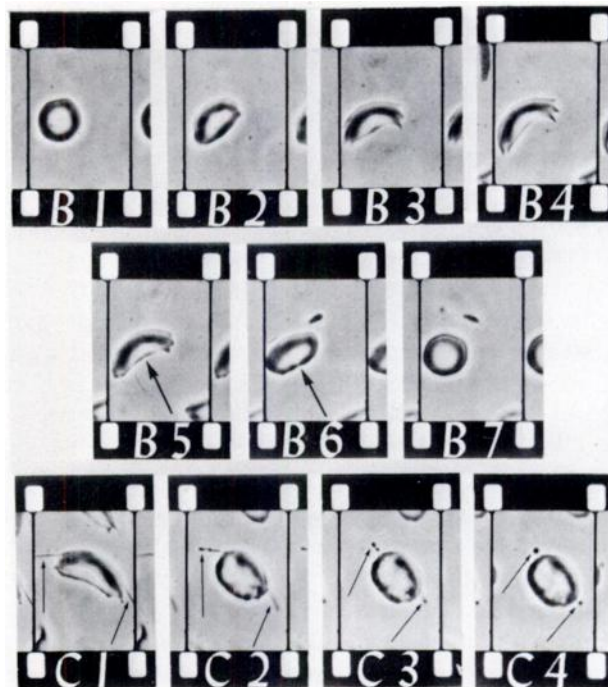


Fig. 2. Montage of seven frames from film sequence B and four frames from film sequence C.

cytes under controlled conditions. Technical manipulations, such as the introduction of needle probes for measurement of O_2 tension and temperature, could be done without escape of cells from the field of vision or interruption of photography.

Erythrocytes were fixed within the chamber while in their deoxygenated state, after progressive increments of oxygenation, and finally while in their fully oxygenated state by the progressive infusion of 1% buffered glutaraldehyde into the chamber. The cells were then recovered from the chamber and remained in fixative for about 20 hr. The cells were then transferred to an aluminum foil disc, air dried, attached to a specimen mount stub with conductive adhesive (Alkadag), and placed in a vacuum evaporator. The specimen was coated with a thin layer of gold and was examined in a Cambridge Stereoscan MkII microscope.

RESULTS

Frames from four film sequences (A-D) have been selected and arranged in montage form. Figure 1 is a montage of 12 frames from film sequence A. These cells had been previously sickled and unsickled five times and were then exposed to 100% nitrogen for 12 min. Frames A-1 to A-6 were selected from the movie taken during the subsequent 1 hr period of deoxygenation. The first noticeable cell change was disappearance of the "flicker" phenomenon. This phenomenon characterizes normal red cells seen in phase-contrast cinemicrophotography and has been termed by Bessis¹¹ as "the intracellular vibratory or scintillating movement displayed by mature erythrocytes." As the five cells (in brackets) undergo sickling (large arrows), spicules and angulations appeared. Veil-like membranous structures that are almost devoid of hemoglobin can be seen in frame A-6, which also shows that two of the cells failed to sickle.

Following oxygenation (frames A-7 to A-12), transition from sickled to disc form occurred. The small arrows point to one of the cells that showed the streaming of hemoglobin around the periphery leading to the formation of a round cell (frame A-12). This was followed by return of the flicker phenomenon. Some cells (see circle, frame A-12) did not regain their original discoid shape and were noted to be permanently deformed into thorny-appearing spheres (acanthocytes).

Figure 2 is a montage of seven frames from film sequence B and four frames from film sequence C. Frames B-1 to B-4 show the transformation from a biconcave to a fully sickled cell by deoxygenation. Frame B-4 shows a sickled cell with a brassy consistency and a surface with linear streaks, shadow, and reflections. Such surface detail can best be seen in the photograph taken with the scanning beam electron microscope (Fig. 4A). Completion of sickling required 10–12 sec after the first noticeable deformation of the cell. If the onset of sickling was taken as that point when flicker disappeared, then the process lasted about 15 sec. Oxygenation (frames B-5 to B-7) was followed by a progressive retraction of spicules into the body of the cell and hemoglobin flowing (large black arrows, frames B-5, 6) along the cell margin causing it to become round (frame B-7). This cell returned to its original form without fragmentation or permanent deformation. The lowermost four frames are from film sequence C during which a previously deoxygenated sickled cell was exposed to oxygen. The first frame (C-1) shows two projections (small black arrows) at each extremity of the cell. With oxygenation the filaments retracted toward the body of the cell; they were then seen to become flexible and wobbly with beadlike swellings resembling the myelin bodies previously described by Bessis¹² as "cylindrical projections, sinuous

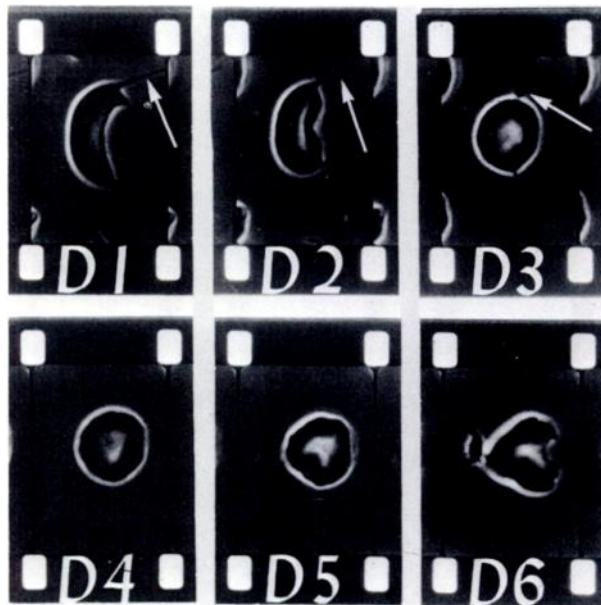


Fig. 3. Montage of six frames from film sequence D.

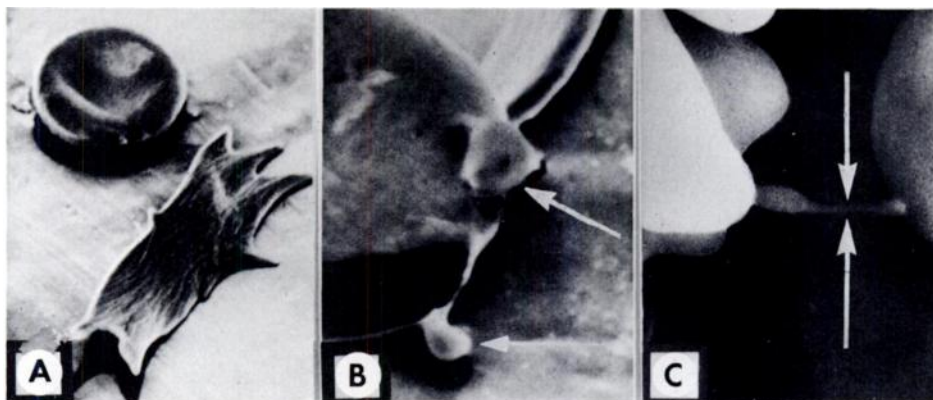


Fig. 4. Scanning beam electron microscopy (SBEM) of HbSS erythrocytes. (A) Surface detail of sickled erythrocyte (below) can be compared with normal biconcave cell (top). $\times 10,000$ (B) Arrows point to spherical bodies of a partially unsickled cell. $\times 10,000$. Enlarged $2\times$. (C) Arrows point to pinching of club-shaped process as it is resorbed into body of cell during unsickling. $\times 10,000$. Enlarged $4\times$.

rods or sometimes filaments with regular beadlike swellings." The spherules contained hemoglobin, as evidenced by absorption of light in the Soret band, and eventually detached from the cell. Frames C-3 and C-4 show two very large myelin bodies (small black arrows) attached to the mother cell by thin filaments.

Figure 3 is a montage of six frames from film sequence D. Frames D-1 to D-3 show the unsickling process in a dolphinlike sickled cell. The longer spicules formed spherules on retraction into the body of the cell. The large white arrows point to one of these. Deoxygenation of the same cell (frames D-4 to D-6) produced a pattern of deformity (frame D-6) that is distinctly different from the dolphinlike deformity seen on frame D-1 during the previous sickling of this cell. This sequence demonstrates the lack of a cellular plastic "memory" during the repetitive sickling of a cell.

Scanning beam electron microscopy (SEM) of cells fixed during the unsickling process revealed cells such as the one in Fig. 4, which shows a portion of a round cell with two somewhat spherical bodies attached to the surface (arrows) of this partially unsickled cell.

Figure 4C shows portions of three cells (magnification $10,000\times$), one of which has a long club-shaped process that may have been undergoing resorption into the body of the cell. Pinching one of the processes (see arrows) appeared to be taking place in one or two areas. This structure presumably represents an intermediate form in the course of microspherule formation.

Cells subjected to repetitive oxygenation and deoxygenation with final fixation in the oxygenated state, when examined by SEM, were noted to have become deformed in the shape of acanthocytes (Fig. 5A).

Figure 5C shows an SEM of the surface of a deoxygenated sickled cell with a thin veil-like area of cell membrane (see arrow). Although unsickling did not

lead to the loss of these veil-like structures, it is apparent that if the veil-like membrane were lost (e.g., due to mechanical trauma) the resulting cell would be constrained to assume a bipolar shape following reoxygenation. Such a cell can be seen in Fig. 5B. This cell morphologically resembles an "irreversibly sickled cell." The hemoglobin content of such cells would be essentially intact, as is true of irreversibly sickled cells.¹³

DISCUSSION

The experimental design of these studies was such that we believe our in vitro conditions resemble some features of the in vivo state.

As has been previously noted, there was variability in the propensity to sickling of different sickle cells from a given individual and subjected to the same in vitro environment. Even with prolonged hypoxia, a few cells remained unsickled; some developed spicules that varied in size, shape, number, and distribution, while others became deformed into "holly-leaf" cells, "burr" cells, and acanthocytes.

The observation that repetitive sickling of a single cell did not result in an identical sickle deformity suggests a reasonably wide range of molecular freedom within the cell and a lack of plastic memory in the cell membrane. The variable deformities were seemingly a result of haphazard and unpredictable polymerization of the hemoglobin molecules during deoxygenation.

We also observed that although disk-to-sickle-to-disk transformation may

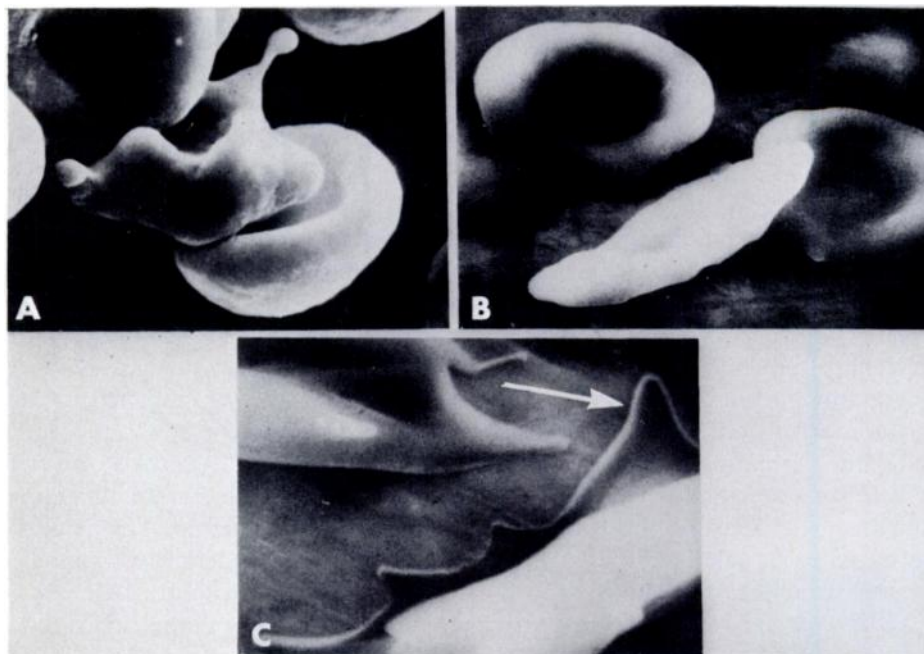


Fig. 5. SBEM of HbSS erythrocytes. (A) Deformed by repetitive oxygenation and deoxygenation. (B) Sickled cell: arrow points to veil-like area of cell membrane. (C) Oxygenated cell morphologically resembling the "irreversibly sickled cell."

occur without apparent membrane loss or distortion, it often was accompanied by the shedding of microspherules that sometimes produces permanent cell deformation. The deformed cell is believed to have been "scarred" by the membrane loss accompanying repetitive cycles of sickling and unsickling. Attempts to cause such cells on deoxygenation to develop long, thin spines or projections failed. On resickling, such cells developed a variety of deformities, but never the elongated, long-spicule shape found following the initial deoxygenation. Nevertheless, microspherule formation and loss was observed during reoxygenation of these cells. This observation suggests that cell membrane plasticity plays a significant role in determining the type of deformity that cells may develop. It may be that the acanthocytes, burr cells, and holly-leaf cells known to occur *in vivo*¹⁴ are deformities taken by cells that have previously undergone membrane loss during repetitive sickle-unsickle cycles while circulating.

Selection of frames from movie sequences limits our ability to properly depict phenomena, such as red cell flicker. Following deoxygenation the loss of flicker always preceded cell deformation. The loss of cell flicker presumably results from polymerization of hemoglobin molecules subadjacent to the cell membrane. It suggests existence of very close contact between Hb molecules and cell membrane. On reoxygenation, flicker reappeared only after the cell had regained a discoid contour. Therefore, loss of cell flicker may indicate an early stages of "molecular sickling" predominantly involving the regions subjacent to the cell surface. Electron microscopic studies of cells fixed soon after the loss of red cell flicker may reveal the beginning of hemoglobin aggregation and/or rod formation.¹⁵⁻¹⁷

The time of oxygen desaturation required for sickling is believed to be shorter than previously reported.^{7,8} The rate of sickling following sudden deoxygenation has been found to be less than 0.12 sec,⁹ a duration that would allow large numbers of sickled cells to form *in vivo*. Our studies revealed that once the sickling process begins (the disappearance of red cell flicker), it lasts for about 15 sec. We also found that the duration of the unsickling process was identical to that of sickling and that it did not vary among different cells nor within the same cell during repetitive cycles of sickling and unsickling.

The levels of oxygen saturation and oxygen tension that exist *in vivo* are, for the most part, above the level required to produce very high numbers of sickled cells. However, repetitive sickling-unsickling may result in cells so deformed as to impair their plasticity even when oxygenated, thus initiating the sequence of stagnation, deoxygenation, further sickling, thrombosis, infarction, etc.¹⁸ Furthermore, the circulating deformed cells could be recognized as abnormal by the reticuloendothelial system with subsequent erythrophagocytosis.

Cell fragmentation and membrane loss demonstrated in the sickle cell may be a process common to red cell death under a wide variety of pathologic circumstances, as well as an occurrence inherent to the aging of the normal cell.

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