Effects of Centrifugation on Transmembrane Water Loss from Normal and Pathologic Erythrocytes (42846)

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Abstract. **Plasma ^{125}I-albumin was used as a marker of extracellular dilution in order to study the effect of high-speed centrifugation on transmembrane water distribution in several types of human red cells, including normal (AA), hemoglobin variants (βA, AS, SC, βS, and SS), and those from patients with hereditary spherocytosis. SS and AA erythrocytes were also examined for changes in intracellular hemoglobin concentration of three different density fractions and with increasing duration of spin. The minimum force and duration of centrifugation required to impair water permeability were found to vary with the red cell type, the anticoagulant used (heparin or EDTA), the initial hematocrit of the sample centrifuged, as well as among the individual erythrocyte fractions within the same sample. When subjecting pathologic erythrocytes to high-speed centrifugation, the ^{125}I-albumin dilution technique can be used to determine whether the centrifugation procedure has led to an artifactual red cell water loss and to correct for this when it does occur. An abnormal membrane susceptibility to mechanical stress was demonstrated in erythrocytes from patients with hereditary spherocytosis and several hemoglobinopathies.** [P.S.E.B.M. 1989, Vol 190]

High levels of centrifugal force can stratify erythrocytes according to cell density (1), and centrifugation has been used to study intracellular characteristics of erythrocytes (2–4). The use of a density gradient reduces the centrifugal force required, allows density equilibrium to be reached in a shorter centrifugation time, and gives a sharper fractionation for the same force (5). Hence, centrifugation through a density gradient has also been used to study erythrocyte fractions (6–8). The red blood cell is highly deformable; one of the determinants of this deformability is the viscoelastic properties of its membrane. Progressive increases in deforming stress may reach a point when the red cell membrane becomes viscoplastic and irreversible membrane deformation occurs (9). Therefore, the mechanical stress due to high-speed centrifugation may cause permanent alterations in membrane characteristics, e.g., the maintenance of intracellular water content.

In order to test this possibility, experiments were performed to determine the effect of high-speed centrifugation on transmembrane water distribution in varied types of human red cells, including normal (AA), several forms of hemoglobin variants heterozygous β thalassemia, heterozygous S, double heterozygous S and C, double heterozygous for β thalassemia and S, homozygous S (βA, AS, SC, βS, and SS, respectively), and erythrocytes from patients with hereditary spherocytosis (HS). SS and AA red cells were also examined for differences in intracellular hemoglobin (Hb) concentration of three different density fractions with increasing duration of spin. We found that the minimum force and duration of spin required to alter intracellular water content vary with the red cell type, the anticoagulant (heparin or EDTA) and the hematocrit, as well as among the individual erythrocyte fractions within the same sample.

Materials and Methods

Fresh blood was obtained from individuals with proven AA, βA, AS, SC, βS, SS, and HS erythrocytes. All subjects gave informed consent. The research was approved by the Human Investigation Committees of

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Columbia-Presbyterian Medical Center and Harlem Hospital Center. No person had been transfused in the preceding 4 months. Two samples, one with heparin (approximately 1.4 USP units/ml blood) and the other with EDTA (approximately 1.5 mg/ml blood) as anticoagulant, were obtained whenever possible. All ultracentrifugation procedures were performed within 12 hr of venesection.

To each sample of blood, ^{125}I-albumin (Mallinckroft, Inc., St. Louis, MO) was added to give a final concentration of approximately 0.5 μCi/ml blood. After gentle mixing for 5 min, the sample was centrifuged at 1000 rpm at 25°C for 5 min (average 1500*g*) in an International Equipment Co. (Needham Heights, MA) centrifuge and two aliquots of 0.5 ml each were collected from the top of the sample for the determination of the initial plasma ^{125}I concentration (C_i) in duplicate.

By removing calculated amounts of plasma, each sample of blood was adjusted to two hematocrit levels: a high hematocrit of 40–45% and a low hematocrit of 30–35%. Aliquots of these samples were transferred to cellulose nitrate centrifuge tubes and centrifuged at 4°C in a Spinco SW 50.1 swinging bucket rotor (Beckman/Spinco) at 50,000 rpm (average 240,000*g*) for 30, 60, 120, and 180 min. At the termination of each high-speed centrifugation, two aliquots of 0.5 ml of plasma were removed from each tube for the determination of postcentrifuge ^{125}I radioactivity (C) in a well-type scintillation counter (Packard Instrument Co., Downers Grove, IL). The degree of transmembrane water egress can be estimated from the percentage of decrease in plasma radioactivity following high-gravity centrifugation.

In order to determine whether the change in transmembrane water distribution following high-speed centrifugation is reversible, some of the blood samples were resuspended in the cellulose nitrate tubes after 50,000 rpm centrifugation without removing the plasma. The plasma was then collected in duplicate after further centrifugation at 1500*g* at 25°C for 5 min. With the use of a tube cutter, the packed red cells from some SS and AA samples after 50,000 rpm centrifugation for 30 to 180 min were divided into three approximately equal fractions (top, middle, and bottom) and resuspended in buffered saline (pH 7.4). Care was taken to exclude the white cells and platelets. The same fractions from blood samples spun for 30 and 60 min were pooled in one tube and those spun for 120 and 180 min in another tube. These erythrocyte fractions were washed three times with the buffered saline and packed in the final washing in a Spinco centrifuge at 30,000 rpm (average 85,000*g*) for 15 min. The packed erythrocytes thus obtained were lysed with the use of toluene and glass beads and finally ultracentrifuged at 85,000*g* for an additional 15 min. The hemoglobin solution was removed by puncturing the bottom of the cellulose nitrate tube; measurements were made on Hb concentration (as cyanmethemoglobin) and ^{125}I radioactivity. The ^{125}I radioactivity in the remaining membrane debris was also determined.

In order to explore the effect of variable relative centrifugal force (RCF), aliquots of SS blood samples collected in heparin and adjusted to a hematocrit of 30–35% were spun for 20,000, 30,000, 40,000, and 50,000 rpm in the SW 50.1 rotor. The ^{125}I radioactivity in the resultant plasma was again measured and compared with the initial plasma ^{125}I radioactivity as described above.

Results

The present experiments were designed to investigate the effect of high-speed centrifugation on the translocation of water across the red cell membrane by using plasma ^{125}I-albumin as a marker for extracellular dilution. Counting of membrane debris and intracellular contents (Hb solution) after lysis of erythrocytes showed no significant amounts of ^{125}I, supporting the assumption that ^{125}I-albumin remained extracellularly in the plasma. The plasma did not contain detectable amounts of hemoglobin (cyanmethemoglobin method) after ultracentrifugation of the blood samples, indicating the absence of significant hemolysis.

AA red cells had no detectable transmembrane water loss after high-speed centrifugation at 50,000 rpm (mean centrifugal force 240,000*g*) for up to 120 min (Table I). Following 180 min of spinning, however, there was a slight decrease of plasma ^{125}I-albumin concentration, indicating its dilution by water leaving the red cells. For AS red cells, this water loss became detectable at 120 min, but the degree was still very slight (approximately 1% dilution of plasma ^{125}I-albumin). SS red cells showed significant water loss following only 30 min of high-gravity centrifugation, and the plasma ^{125}I-albumin was diluted by nearly 10% after a 3-hr high-speed centrifugation in heparinized samples. βS red cells had slightly greater water loss than SS red cells, especially at the shorter centrifugation times, and the greatest water loss was found in the red cells obtained from patients with HS. The behaviors of SC and βA red cells were between those of SS and AS red cells. The extent of plasma ^{125}I dilution is generally in the order HS > βS > SS > SC > βA > AS > AA. Lowering the initial hematocrit from 40 to 45 to 30 to 35% and increasing the centrifugation time to 3 hr increased the ^{125}I dilution in SS and AA samples so that SS $\simeq$ βS and AA $\simeq$ AS.

For each of the seven groups of red cells examined (SS, βS, SC, AS, βA, AA, and HS), samples anticoagulated with heparin were affected by high-speed centrifugation more than those treated with EDTA (Table I, $P < 0.001$). In each group of red cells, the degree of

dilution of ^{125}I-albumin was greater with the lower initial hematocrit (Table I, $P < 0.001$).

Measurement of plasma ^{125}I in tubes resuspended following high-gravity centrifugation yielded the same results as their duplicate tubes centrifuged without being resuspended.

The lysates of the three fractions of SS as well as AA erythrocytes showed a progressive increase in Hb concentration toward the bottom of the tube (Fig. 1). Increasing the duration of centrifugation raised the intracellular Hb concentration in the middle and bottom fractions of SS cells and the bottom fraction of AA cells. The middle and bottom fractions of SS erythrocytes had higher Hb concentrations than their AA counterparts (Fig. 1).

At a constant centrifuging duration of 1 hr, centrifugal speeds up to 20,000 rpm (or RCF for the bottom fraction at 52,000*g*) did not cause detectable water loss from SS cells (Table II). Further increases in RCF led to a progressive increase in transmembrane water loss and dilution of plasma ^{125}I-albumin.

Discussion

This investigation was based on the assumption that ^{125}I-albumin remains in the plasma and does not enter the red cell via its membrane; therefore, a decrease in plasma ^{125}I concentration reflects a dilution due to the extrusion of intracellular water. ^{125}I-Albumin radioactivity was not detectable in washed membrane debris (i.e., no specific binding to membrane component) or intraerythrocytic content. There was no significant dilution of ^{125}I activity due to hemolysis as judged from the lack of plasma hemoglobin.

In all types of erythrocytes studied, the samples collected with heparin as the anticoagulant had greater water loss than those collected in EDTA, for the same RCF and duration of centrifugation; this indicates that these anticoagulants have different effects on the water content of the red cell.

The different influences of high-speed centrifugation on the membranes of normal erythrocytes, erythrocytes of various hemoglobinopathies (SS, βS, SC, βA, and AS), and HS erythrocytes suggest a variability in the potential of these red cell membranes to "leak" water (Fig. 2). HS is a disease which is known to affect the red cell membrane (10, 11), and our findings indicate a greater "leakiness" in HS red cells than in those of the hemoglobinopathies studied. SS erythrocytes have been shown to have membrane damage (12–14), and there appears to be a hemoglobin-membrane interaction (15). Our results suggest the presence of membrane abnormality also in other S hemoglobinopathies; the defect has the order; βS > SS > SC > AS. It is to be noted that β thalassemia as such also has some water loss.

The irreversible nature of the water loss with centrifugation suggests a corresponding loss of cell solutes. The bottom fraction of SS erythrocytes suffered the greatest water loss following high-speed centrifugation, and it has been shown to have the lowest cation concentration per kg cell water (6) or per kg RBC (8, 16, 17). Differences in cation concentrations between AA and SS erythrocytes have also been documented (18).

The difference in water loss from different fractions of erythrocytes subjected to high-speed centrifugation may be related to the variations in centrifugal force depending on the location of the cells in the tube. The centrifugal force (RCF) can be calculated as $\omega^2 x/980$, where ω is the angular velocity and equals $2\pi n/60$, n being the number of rpm, and x is the radial location

Table I. Effect of Centrifugation[a] on Percentage of Decrease in Plasma Radioactivity[b]

RBC	Hct	Heparin				EDTA			
		30 min	60 min	120 min	180 min	30 min	60 min	120 min	180 min
AA (n = 9)	H[c]	0	0	0	0.94 (0.19)	0	0	0	0.17 (0.12)
	L	0	0	0	1.36 (0.18)	0	0	0	0.41 (0.25)
AS (n = 4)	H	0	0	0.67 (0.06)	1.36 (0.06)	0	0	0.54 (0.08)	1.09 (0.11)
	L	0	0	0.80 (0.03)	1.65 (0.12)	0	0	0.63 (0.10)	1.22 (0.14)
SS (n = 14)	H	1.08 (0.09)	2.18 (0.19)	4.65 (0.45)	8.90 (0.57)	0.36 (0.20)	1.02 (0.17)	2.46 (0.63)	4.65 (0.89)
	L	1.34 (0.23)	2.58 (0.31)	5.24 (0.50)	9.78 (0.67)	0.64 (0.24)	1.48 (0.28)	3.04 (1.03)	5.43 (1.04)
SC (n = 5)	H	0	1.37 (0.21)	2.82 (0.27)	4.26 (0.21)	0	0.91 (0.09)	2.32 (0.38)	3.59 (0.32)
	L	0.07 (0.02)	1.55 (0.21)	3.32 (0.24)	5.04 (0.08)	0	1.08 (0.11)	2.71 (0.35)	4.04 (0.16)
βS (n = 5)	H	1.81 (0.14)	3.66 (0.49)	7.12 (0.86)	8.81 (0.51)	1.35 (0.11)	2.90 (0.23)	5.47 (0.69)	6.62 (0.49)
	L	2.06 (0.26)	4.14 (0.49)	7.62 (0.82)	9.28 (0.75)	1.59 (0.13)	3.29 (0.40)	5.94 (0.59)	7.26 (0.63)
βA (n = 3)	H	0	0.56 (0.11)	1.52 (0.15)	3.16 (0.22)	0	0.39 (0.07)	1.10 (0.06)	3.07 (0.29)
	L	0	0.66 (0.08)	1.77 (0.09)	3.67 (0.49)	0	0.51 (0.03)	1.52 (0.08)	3.34 (0.30)
HS (n = 2)	H	2.40 (0.57)	5.57 (0.62)	8.62 (0.07)	11.16 (0.49)	ND	ND	ND	ND
	L	3.00 (0.15)	6.85 (0.71)	9.06 (0.06)	12.81 (0.98)	ND	ND	ND	ND

[a] 240,000*g* (50,000 rpm) for periods shown.
[b] Values are mean ± SD.
[c] AA, homozygous hemoglobin A; AS, heterozygous hemoglobin S; SS, homozygous hemoglobin S; SC, double heterozygous hemoglobin S and C; βS, double heterozygous β thalassemia and hemoglobin S; βA, heterozygous β thalassemia; HS, hereditary spherocytosis; H, high hematocrit = 40–45%; L, low hematocrit = 30–35%; ND, not done.

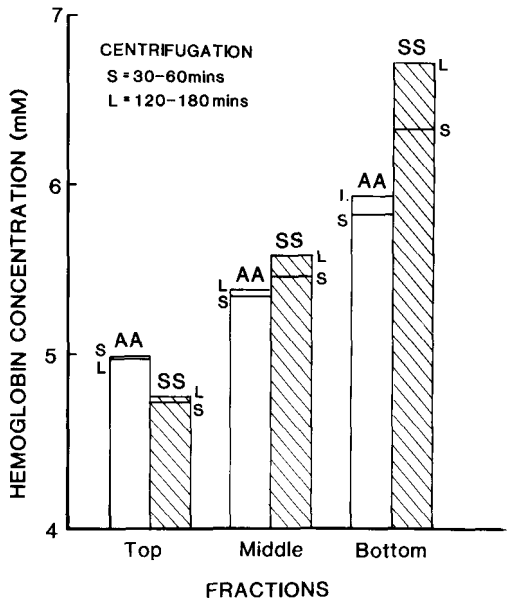

Figure 1. Hemoglobin concentration of lysates from density-fractionated SS and AA erythrocytes that had been centrifuged for 30 to 60 or 120 to 180 min. Centrifugal force = 240,000*g*.

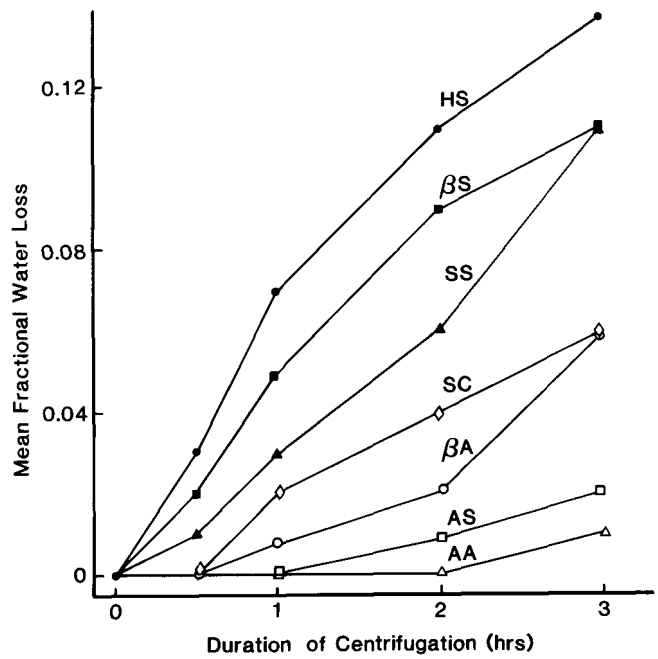

Figure 2. Mean fractional red cell water loss in patients with HS, patients with various forms of hemoglobinopathies (βS, SS, SC, βA, and AS) and control subjects (AA). Initial hematocrit = 40–45%. Heparin was used as an anticoagulant. The red cell water loss was calculated from data on percentage of decrease in plasma ^{125}I activity in Table I (see Appendix).

Table II. Percentage of Decrease in Plasma ^{125}I-Albumin Radioactivity in SS Blood Centrifuged at Various Speeds for 1 Hr[a]

Experiment	Centrifugal speed (rpm)			
	20,000	30,000	40,000	50,000
1	0	0.05	0.87	2.61
2	0	0.04	0.68	2.37
3	0	0.01	0.59	2.11
4	0	0.04	0.93	2.87
5	0	0.02	0.51	2.20
Mean	0	0.03	0.72	2.43
SD		0.02	0.18	0.31

[a] Rotor = SW 50.1; hematocrit = 30–35%.

of the erythrocytes in the tube during centrifugation. At a constant spin frequency (e.g., n = 50,000 rpm), the bottom-fraction erythrocytes are subjected to a higher gravity force than the middle or top fractions. At a given hematocrit and duration of centrifugation, the RCF increases linearly from the top toward the bottom of the tube. The percentage of dilution of plasma ^{125}I-albumin and RBC water loss can be correlated with the average RCF (Table II, $r = 0.958$, $P < 0.05$). When suspensions of red cells with a lower initial hematocrit are centrifuged, the packed red cell column occupies a shorter height in the tube and hence the red cells are subjected to a higher average gravity force. This could explain the greater water loss from erythrocytes of the lower hematocrit suspensions for all groups studied.

The centrifugation process is a form of mechanical stress, possibly an exaggeration of the mechanical stresses encountered by red cells during their passage through the microcirculation. These stresses may possibly act on the red cell membrane to cause water and solute losses and an increase in mean corpuscular hemoglobin concentration (MCHC) with age in AA cells, and even prematurely in susceptible erythrocytes from patients with HS and several other hemoglobinopathies.

The present investigation indicates that high-speed centrifugation can cause water loss from erythrocytes, and the amount of water loss varies with the cell type, the anticoagulant used, the hematocrit of the sample, and the force and duration of centrifugation. When centrifuging red cell suspensions at high speed, one should take into account these variables in order to determine whether the centrifugation has caused transmembrane water leakage and attendant alterations in MCHC. The duration of centrifugation should be kept at the shortest time required to effect the experimental purpose. Centrifugation through a density gradient (5) may help to achieve this, although the effect of the density gradient on red cell water and attendant electrolyte loss would have to be examined. The ^{125}I-albumin dilution technique described here allows the determination of whether the centrifugation procedure has led to an artifactual red cell water loss and the correction for this loss when it occurs (see Appendix). Furthermore, this provides a method for determining the integrity of the red cell membrane in the face of imposed stress.

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Appendix: Calculation of Fractional Red Cell Water Loss from Dilution of Plasma ^{125}I-Albumin

For a given blood sample, let the initial values of fractional plasma volume, plasma ^{125}I radioactivity, and red cell water content (determined by desiccation to constant weight) by V_{pi}, C_i, and W_i, respectively, and the corresponding values after ultracentrifugation by V_p, C, and W, respectively. Since a constant amount of ^{125}I-albumin remains in the plasma,

$$\frac{V_p}{V_{pi}} = \frac{C_i}{C} \tag{A1}$$

the fractional gain in plasma volume (ΔV_p) is

$$\Delta V_p = \frac{V_p - V_{pi}}{V_{pi}} = \frac{C_i - C}{C} \tag{A2}$$

Whenever there is transmembrane loss of cell water, the loss in cell water content is equal to the gain in plasma volume

$$W_i - W = V_p - V_{pi} \tag{A3}$$

The fractional loss of cell water (ΔW), which is defined as $(W_i - W)/W_i$, can be expressed as

$$\Delta W = \Delta V_p(V_{pi}/W_i) \tag{A4}$$

Since

$$V_{pi} = 1 - h_i \tag{A5}$$

where h_i is the initial fractional hematocrit, Eq. (A4) can be written as

$$\Delta W = \Delta V_p(1 - h_i)/W_i \tag{A6}$$

or

$$\Delta W = (1 - h_i)(C_i - C)/CW_i \tag{A7}$$