JCI The Journal of Clinical Investigation

Hemoglobin CC disease: rheological properties of erythrocytes and abnormalities in cell water

John R. Murphy

J Clin Invest. 1968;47(7):1483-1495. https://doi.org/10.1172/JCI105842.

Research Article

Suspensions of erythrocytes from patients with hemoglobin (Hb) CC disease showed an increased viscosity and decreased filterability suggesting a less deformable cell. Hemolysates prepared from Hb CC erythrocytes had an increased viscosity compared with hemolysates of normal cells, suggesting that the increased viscosity of Hb CC cells in serum was the result of an increased internal viscosity of the cell. These abnormal rheological properties of Hb CC erythrocytes were associated with a decreased content of cations and an abnormality of cell water. The fraction of the cell volume, which is water in Hb CC cells, was 95.5% of normal. The amount of cell water in Hb CC cells available for osmotic equilibrium, termed solvent water, was only 67% of that in normal cells. The smaller amount of solvent water in Hb CC cells indicates a greater amount of water bound to protein.

Altered rheological properties of erythrocytes with Hb AA, CC, and SC also were observed during pregnancy. Suspensions of erythrocytes in serum or plasma from pregnant patients resulted in an increased viscosity compared with suspension in serum or plasma from nonpregnant individuals. An increased viscosity during pregnancy is consistent with the increased severity of the hemolytic anemia in patients with these hemoglobinopathies during pregnancy. The studies reported here suggest that in Hb CC disease the mechanism of erythrocyte destruction, […]

Find the latest version:

https://jci.me/105842/pdf

Hemoglobin CC Disease: Rheological Properties of Erythrocytes and Abnormalities in Cell Water

John R. Murphy

From the Department of Medicine, University Hospitals and Case Western Reserve University, Cleveland, Ohio 44106

Abstract Suspensions of erythrocytes from patients with hemoglobin (Hb) CC disease showed an increased viscosity and decreased filterability suggesting a less deformable cell. Hemolysates prepared from Hb CC erythrocytes had an increased viscosity compared with hemolysates of normal cells, suggesting that the increased viscosity of Hb CC cells in serum was the result of an increased internal viscosity of the cell. These abnormal rheological properties of Hb CC erythrocytes were associated with a decreased content of cations and an abnormality of cell water. The fraction of the cell volume, which is water in Hb CC cells, was 95.5% of normal. The amount of cell water in Hb CC cells available for osmotic equilibrium, termed solvent water, was only 67% of that in normal cells. The smaller amount of solvent water in Hb CC cells indicates a greater amount of water bound to protein.

Altered rheological properties of erythrocytes with Hb AA, CC, and SC also were observed during pregnancy. Suspensions of erythrocytes in serum or plasma from pregnant patients resulted in an increased viscosity compared with suspension in serum or plasma from nonpregnant individuals. An increased viscosity during pregnancy is consistent with the increased severity of the hemolytic anemia in patients with these hemoglobinopathies during pregnancy. The studies reported here suggest that in Hb CC disease the mechanism of erythrocyte destruction, splenic sequestration, results from the increased viscosity, and less deformability of the erythrocyte with on increased internal viscosity.

Preliminary reports of these studies were presented at the First International Conference on Hemorheology, Riykjavik, Iceland, July 1966, and at the American Society of Hematology, 1966. *Blood* **28:** 1017, 1966.

Received for publication 2 October 1967 and in revised form 4 December 1967.

INTRODUCTION

Hemoglobin (Hb) CC disease has been characterized as a mild anemia with splenomegaly. The anemia is the result of a shortened survival of the erythrocyte as well as a relative lack of erythrocyte production. Ferrokinetic studies in Hb CC disease have demonstrated an inadequate compensatory increase in erythrocyte production in the presence of anemia (2, 3). Ashby and ^{51}chromium survival studies indicate a moderate shortening of erythrocyte survival (3, 4, 5). The mild anemia usually is not associated with symptoms or crises as seen in other hemolytic anemias (1, 6). The absence of aplastic crises probably is related to the mild shortening of the life span of the erythrocyte with Hb CC. The molecular defect in hemoglobin C is due to the substitution of the amino acid, lysine, for glutamic acid in the β-chain at the same site valine replacement occurs in Hb S (7). The mechanism whereby Hb C results in a shortened survival of the erythrocyte in the homozygous state (Hb CC) has not been defined. Although the heterozygous state, Hb AC, has not been associated with clinical abnormalities, the combination of hemoglobins S and C results in anemia and a particularly severe clinical picture during pregnancy (1).

The spleen is invariably enlarged in Hb CC disease (1, 6). Survival studies of the cells labeled with ^{51}chromium indicate splenic sequestra-

tion (8). Splenectomy in some patients with Hb CC disease has resulted in an increase in the hematocrit to normal and a decrease in the reticulocytes. The histological description of the spleen in Hb CC disease was similar to that observed in hereditary spherocytosis (4, 9, 10).

The studies reported here deal with the rheological properties of erythrocytes that contain Hb C, including packing in Wintrobe hematocrit tubes, viscosity as determined in a cone-plate viscometer, and deformability of the erythrocyte as indicated by filtration through microfilters of uniform pore size. Erythrocytes containing Hb CC were compaired with cells that contained Hb AA, AC, and SC. The increased viscosity of Hb CC was associated with a large fraction of water bound to Hb, decreased cation content of the cells, and a smaller amount of free or solvent water. The increased viscosity and decreased deformability of erythrocytes containing Hb CC suggests that the spleen sequesters these cells on the basis of the abnormal rheological properties. Increased viscosity and decreased filtration of Hb CC blood has also been described by Conley and Charache (11), and attributed to a "precrystalline" state of the intracellular Hb (12).

METHODS

Human venous blood was obtained after an overnight fast, defibrinated, cooled in ice, and centrifuged at 2000 *g* for 20 min at 2–5°C. The serum was removed and the buffy coat was aspirated. The cells were resuspended in serum and the procedure repeated with final suspension in serum at the desired hematocrit. pH adjustments were made by equilibration of the blood with a gas mixture consisting of air plus a variable amount of CO_2 saturated with water vapor at the temperature of the blood at the time of pH adjustment, 25°, or 37°C. All studies were done on the day the blood was obtained and kept at 2–5°C until used to prevent metabolic changes in the cells (13). The rheological properties of cells were not altered by prior refrigeration. Hemoglobin was identified by starch-gel electrophoresis (14) and DEAE-cellulose column separation (15). In studies involving plasma, venous blood was anticoagulated with Na_2 H_2 ethylenediaminetetraacetate (EDTA) and otherwise handled like defibrinated blood.

The microhematocrit method included centrifugation (International microhematocrit centrifuge) for 10 min at 11,500 rpm (13,500 *g*). Red blood cell counts were determined in duplicate in isotonic phosphate-buffered NaCl solutions at pH 7.4 on a "Celloscope" electronic cell counter.[1] The osmotic fragility was performed at pH 7.4 and 25°C as described previously (16). Suspensions of erythrocytes for phase microscopy were fixed in 1% glutaraldehyde in 0.60% phosphate-buffered NaCl as described (16). The rate of erythrocyte packing in Wintrobe hematocrit tubes was determined by centrifugation for various times at 1000 *g*, 25°C, hematocrit $50.0 \pm 0.1\%$ (microhematocrit), and pH 7.4.

Blood viscosity was determined in the Wells-Brookfield cone-plate viscometer (16, 17), model LVT 1/2.[2] Suspensions of erythrocytes in serum for viscosity determination were at a hematocrit of 60% to reduce "plasma skimming" in the instrument and increase the accuracy of the instrument. Several studies of viscosity were carried out on the GDM-viscometer (18), through the courtesy of Dr. Roe E. Wells, Jr., Department of Medicine, Peter Bent Brigham Hospital, Boston, Mass.

Hemolysates for viscosity determination were prepared by four methods: (*a*) osmotic lysis with 10 volumes of water, centrifugation at 16,000 *g* for 2 hr, and reconstitution to a hemoglobin concentration of 30–34 g/100 ml by pressure dialysis, all at 1–5°C; (*b*) and (*c*) extraction three times with 2 volumes of chloroform, or toluene, and centrifugation at 25°C for 20 min at 2000 *g*, followed by a fourth extraction and centrifugation at 1°C for 1 hr at 16,000 *g*; and (*d*) sonic destruction of packed cells at 10°C in a Raytheon 10-KC sonicator for 8 min without subsequent centrifugation. This time was chosen since it resulted in a minimal viscosity. The viscosities of hemolysates were determined at 25°C because there was a progressive increase in viscosity of hemolysates when determined at 37°C that did not occur at 25°C.

An estimation of the deformability of individual erythrocytes was obtained by determining the time required for filtration of a 2-ml suspension of erythrocytes in serum at hematocrit 2%, pH 7.4, 37°C, and 15 cm of H_2O pressure through microfilters of uniform pore size (16). The estimation of cell deformability by filtration was originally suggested by Jandl, Simmonds, and Castle (19) and Nicolau, Teitel, Fotino, Butoianu, and Targar (20). In the filtration studies reported from this laboratory (16) larger and more uniform microfilters were used at lower pressures, as compared to previous studies (19). Temperature and pH have also been shown to influence erythrocyte filtration (16). The pore size, 8.0 to 8.7 and 8.7 to 9.4 μ (SC type),[3] refers to the mean diameter of the pore opening on the surface of the filter and not to the diameter of the channel within the filter. Microscopically, the pores in the SC-type filter are not straight channels but rather are very distorted with smaller minimal diameters than the diameter of the pore opening on the surface of the filter.

The determination of erythrocyte sodium (Na) and potassium (K) was performed as described previously (21). The fraction of cell volume due to water was measured gravimetrically as described by Savitz, Sidel, and Solomon (22). An estimation of the osmotically active

[1] Particle Data, Inc., Elmhurst, Ill.

[2] Brookfield Engineering Laboratories, Inc., Stoughton, Mass.

[3] Millipore Filter Corp., Bedford, Mass.

TABLE I
Hematological Data in Hb CC Disease

Patient	Hematocrit	Hemoglobin	RBC	Reticulocytes	MCHC	MCV	MCH
	%	g/100 ml	$\times 10^6$/cm	%	g/100 ml	μ^3	$\mu\mu g$
1	31.4	12.2	3.94	4.1	38.9	80.0	31.0
2	35.0	13.6	4.92	3.3	38.4	71.2	27.6
3	34.6	13.6	4.94	3.8	36.8	70.0	28.9
4	28.0	10.7	4.32	2.3	38.2	65.0	24.8
5	31.6	11.1	5.50	1.9	35.2	57.5	20.2
6	38.0	15.0	4.85	3.5	39.5	83.0	30.9
7	32.0	12.2	4.30	2.8	38.2	74.4	28.4
8	29.8	10.8	4.14	5.0	36.3	72.0	26.1
9	30.0	12.3	4.25	4.2	41.1	70.5	29.0
Mean	32.2	12.4	4.57	3.6	38.0	71.6	26.9

RBC, red blood cells; MCHC, mean corpuscular hemoglobin concentration; MCV, mean corpuscular volume.

water in the cell was determined as described by Savitz et al. (22), with the following modifications. The procedure was carried out at 25°C, at pH 7.40, by washing cells three times with 10 volumes of phosphate-buffered NaCl, 0.50%, pH 7.4. The change in cell volume in hypotonic media, as compared to the volume in isotonic serum, was determined by measurement of both microhematocrit and change in cell water content, both of which indicate the fraction of cell volume representing osmotically available water or solvent water. All microhematocrit measurements in the determination of cell water and osmotically active water were corrected for trapped plasma by use of ^{131}I albumin as described previously (16). Chloride was determined in Folin-Wu filtrates of cell suspensions and serum by the method of Catlove, Trantham, and Browman (23).

RESULTS

Hematological data. The hematological data for the nine patients studied are shown in Table I. In this study the mean corpuscular volume (MCV) is smaller and the mean corpuscular hemoglobin concentration (MCHC) is greater than reported previously (12). These differences probably are the result of determining the hematocrit by the microhematocrit method with centrifugation for 10 min, in contrast to the usual Wintrobe hematocrit (12). The low MCV, high MCHC, and low MCH are important features of this disease. Fig. 1 shows the mean osmotic fragility curves obtained for the nine patients with Hb CC, 20 normals and 10 patients with hereditary spherocytosis before splenectomy. In Hb CC disease a small per cent of cells with a marked increase in osmotic fragility were not found although microspherocytes in Hb CC disease are seen in the dry blood film (Fig. 2). This is in contrast to the "trail" in the osmotic fragility curve (Fig. 1) seen with hereditary spherocytosis with microspherocytes. Examination of wet preparations of peripheral blood from four patients revealed intracellular crystals in less than 0.01% of the cells. On dry blood films, crystals were present in less than 0.3% of the cells. Examinations by phase microscopy of suspensions of glutaraldehyde-fixed erythrocytes or fresh erythrocytes in serum at 37°C, all from patients with Hb CC disease, shows a marked variation in diameter of flat cells and the absence of target cells which are seen in the dried blood film of fresh cells (Fig.

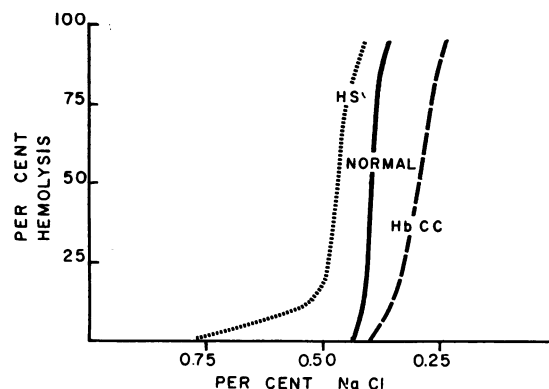

FIGURE 1 Osmotic fragility. The mean osmotic fragility curves for 9 patients with Hb CC disease, 20 normals, and 10 patients with hereditary spherocytosis (HS) before splenectomy. The decreased osmotic fragility of Hb CC erythrocytes is shown with lysis in more hypotonic solution than that of normal cells. The increased osmotic fragility of hereditary spherocytosis blood is shown with lysis occurring in less hypotonic solution than that of normal cells.

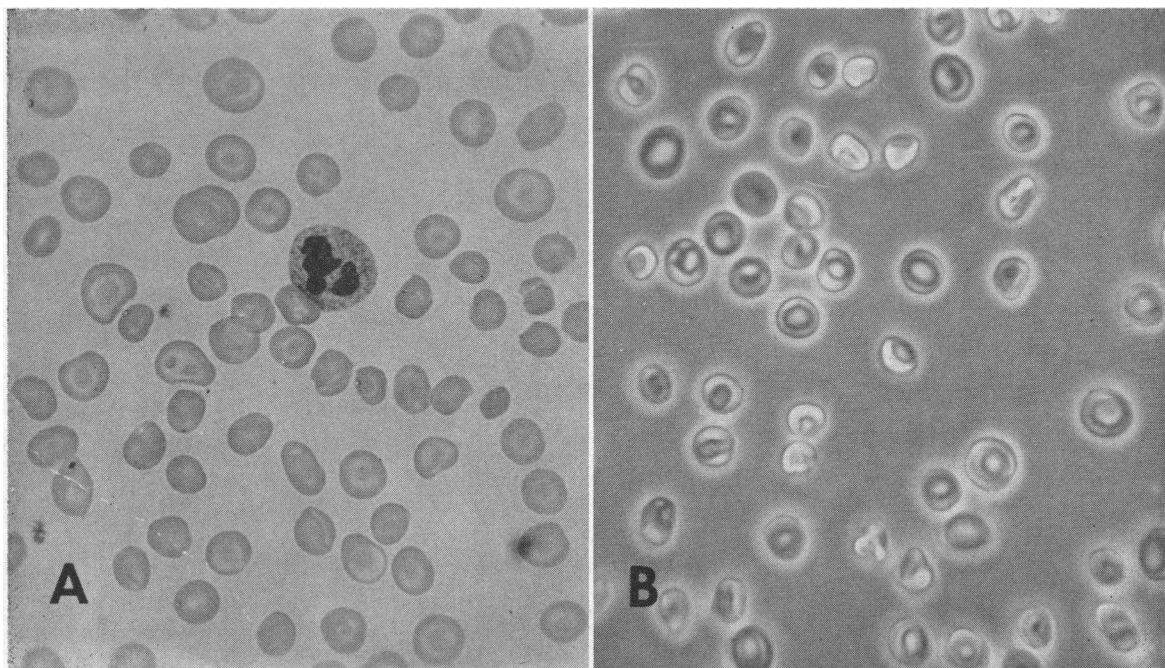

FIGURE 2 *A* and *B* Photomicrographs of Hb CC erythrocytes. *A,* Wrights' stained, dry blood film showing target cells with the more intensely stained central area surrounded by a light area. *B,* phase photomicrograph of a glutaraldehyde-fixed wet preparation of Hb CC cells showing the absence of target cells. The variation in cell size is apparent.

2) but not in dried films of fixed cells. Erythrocytes fixed at 37°C in glutaraldehyde show no change in volume as determined with the aid of ^{131}I albumin (16). It appears that target cells do not exist in wet preparations but are an artifact which occurs when drying fresh cells that have a surface area to volume relationship larger than normal. The larger surface area to volume relationship is also reflected in the decreased osmotic fragility in Hb CC disease.

An estimation of mean surface area of red cells can be calculated from a formula devised by Ponder (24) to determine the volume of cells at the time of osmotic lysis:

$$V_{\text{inc}} = \text{RW}(1/T - 1) + 1$$

V_{inc} = relative increase in volume; R = 0.8, the fraction of water in normal cells that participates in osmotic equilibrium, termed solute water; W = 0.71, the volume fraction of water in normal cells; T = ratio of osmolality resulting in 50% lysis to osmolality of isotonic media. The volume change times MCV equals the mean volume of the cells at the time of osmotic lysis. Using the above equation with the osmotic fragility data, and substituting the corrected values for R and W, which are different in Hb CC disease, and will be described subsequently, the mean volume of normal cells at the time of osmotic lysis was 151 μ^3 and Hb CC cells, 131 μ^3. The smaller volume for Hb CC cells at the time of lysis indicates that the surface area at the time of lysis of the average cell in Hb CC disease was smaller than normal. Examination of the blood films indicates that there was a greater spectrum of cell sizes in Hb CC disease than in normals, an observation which is unexplained.

Erythrocyte packing. Erythrocytes containing Hb CC did not pack as well as normal cells during centrifugation, which suggested that the abnormal cells were less deformable than normal cells. Fig. 3 shows the actual Wintrobe hematocrit values obtained after centrifugation at 1000 *g* for various times for Hb AA, AC, SC, and CC cells at 25°C and pH 7.4, when the hematocrit determined by the microhematocrit at 10 and 15 min was 50.0%. At 4 min in the microhematocrit, Hb CC cells read 1.5–2.0 percentage points higher than the 10 min values, whereas normal cells showed no change after 4 min of centrifugation at 11,500 rpm. Thus

all microhematocrits were centrifuged for 10 min. The packed cell mass of normal erythrocytes centrifuged for 10 min in the microhematocrit centrifuge, 11,500 rpm, contained 2.1–2.3% trapped plasma. Under similar conditions, packed Hb CC cells contained 3.1–4.0% trapped plasma. The hematocrit values were not corrected for trapped plasma except in the calculation of cell water. Correction of the hematocrit values used in calculating the red cell indices would result in a more abnormally low MCV and high MCHC for Hb CC cells.

Viscosity. Erythrocytes containing Hb CC had twice the viscosity of normal erythrocytes (Fig. 4) under identical conditions of hematocrit 60%, 37°C, and at pH 7.40. Charache, Conley, Waugh, Ugoretz, and Spurrell (12) have reported similar differences in viscosity for washed cells at hematocrits greater than 97%. The viscosity of erythrocytes containing Hb AC was somewhat increased. The differences in viscosity were not related to reticulocytosis. Cells with Hb SC had a viscosity between cells with Hb CC and cells with Hb AC. Both Hb CC and Hb AA cells showed no variation in viscosity with changes in oxygenation and similar increases in viscosity at decreasing pH's, 7.0 and 6.8. A decrease in pH or O_2 saturation to result in sickling of cells with Hb SC at a hct of 60%, showed a five- to sevenfold increase in viscosity.

The viscosity of Hb CC hemolysates was consistently 25 to 35% greater than Hb AA at identical hemoglobin concentrations in the range of 30–34 g/100 ml. This is in contrast to other reported studies (12) of Hb CC where the viscosities of Hb AA and Hb CC hemolysates were similar. The viscosity of Hb CC hemolysates at 25°C were only one-fourth that of Hb CC erythrocytes in saline at 25°C at a shear rate of 5.8 sec^{-1}, the Hb concentration in each, 33 g/100 ml. The viscosities of all hemolysates were Newtonian in character, that is, the viscosity was constant (± 10%) at the shear rates studied in the cone-plate viscometer. This was in contrast to the viscosities of cell suspensions which were non-Newtonian in character, that is, dependent on shear rate and increased at lower shear rates (Fig. 4). The viscosities of toluene- or chloroform-extracted hemolysates and hemolysates concentrated by pressure dialysis showed similar differences and were 10–20% less than the viscosities of hemolysates prepared by sonication. The hemolysates prepared by sonication were not centrifuged.

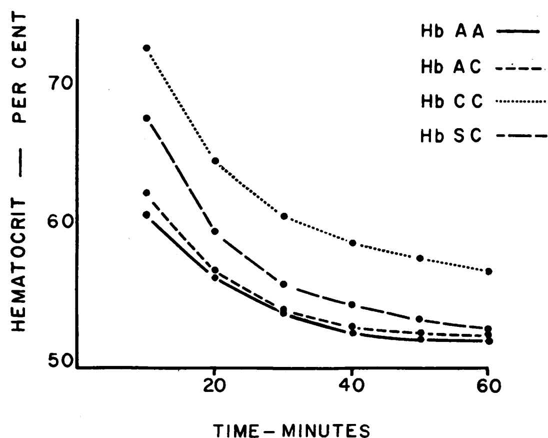

FIGURE 3 Erythrocyte packing. The Wintrobe hematocrit readings for various types of erythrocytes in serum, centrifuged for varying intervals at 1000 *g*, 25°C, and pH 7.4. The microhematocrit value of each sample was 50.0 ± 0.1% after 10 min, or 15 min centrifugation at 13,500 *g*.

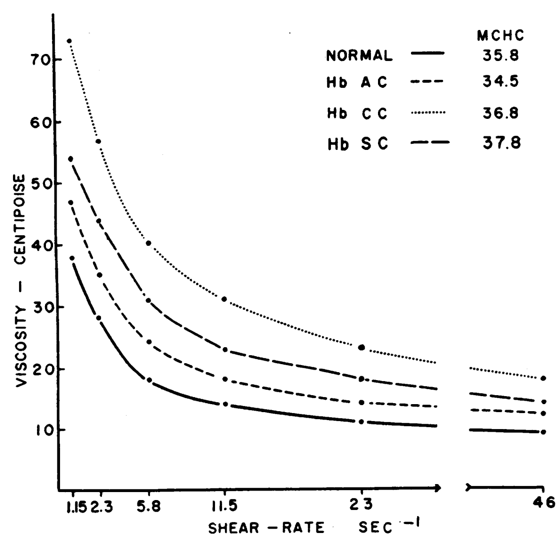

FIGURE 4 Blood viscosity. Viscosity at various shear rates of erythrocyte suspensions in serum at hematocrit 60.0 ± 0.5%, pH 7.4, and 37°C. The curves represent the mean values for 12 normals, 4 patients with Hb AC, 6 patients with Hb CC disease, and 4 patients with Hb SC disease. There was virtually no overlap of data within ± 1 SD.

Filtration. A longer time for filtration (Table II) for erythrocytes from patients with Hb CC disease indicated that these cells were less deformable, or more rigid, than normal cells. Cells with Hb AC showed slight prolongation of the filtration

time. Previous studies (16) have shown that both size and shape, which influence surface area to volume relationships, altered the filtration time. The prolonged filtration time for Hb CC cells, as compared with normal cells, occurred in the presence of both a smaller cell volume as well as a greater surface area to volume relationship. Both of these characteristics of Hb CC cells would be expected to result in a more rapid filtration (16). The longer filtration time of cells with Hb SC, as compared to CC or AC, suggests that the more abnormal shapes of the cells in Hb SC disease also influenced filtration. The effect of Hb C on erythrocyte deformability is also evident by comparing the filtration of cells from patients with C-thalassemia and S-thalassemia. The larger S-thalassemia cells were filtered more rapidly than the smaller C-thalassemia cells. The prolonged filtration time for cells with Hb CC suggests that the cells are less deformable as a result of the increased internal viscosity of the cells as indicated by the hemolysate studies.

Propane studies. The influence of propane on the viscosity of erythrocytes with Hb CC and normal cells was examined to determine if propane would decrease the abnormally high viscosity of Hb CC cells similar to the influence of propane on the viscosity of Hb SS cells. Murayama (25) has reported the reversal or unsickling of sickled erythrocytes and prevention of sickling by equilibration with propane. The effect of propane on Hb CC cells is of interest because the abnormal amino acid substitution in Hb CC is in the same 6th position from the amino terminus of the β-chains in both Hb C and Hb S. The viscosities of both normal and Hb CC cells were increased 30–40% after deoxygenation by equilibration with a mixture of propane and CO_2 at pH 7.4. Deoxygenation of either normal or Hb CC cells by equilibration with nitrogen and CO_2 did not alter viscosity. Equilibration of Hb SC or SS cells with propane resulted in an increased viscosity similar to normal and Hb CC cells and prevented the formation of sickled cells when the cells were deoxygenated as reported by Murayama (25). However, in contrast to that report (25), previously sickled cells, Hb SC or SS, were not "unsickled" by equilibration with propane at 25°C.

TABLE II

Filtration Times in Seconds for Two ml of a Suspension of Cells in Serum to Flow Through a Microfilter at Hct 2%, 37°C, pH 7.4, and 15 cm of H_2O pressure

Subjects (No.)	MCV, μ^3	Time: 8.0–8.7 μ filter	Time: 8.7–9.4 μ filter
		sec	
Normals (10)	90	7.5 (6–10)*	6.9 (5–9)*
Hb CC (6)	81	24,30	9,9
	78	45,46	12,13
	87	24,25	16,20
	54	20,22	14,14
	75	25,27	14,15
	72	20,21	9,10
Hb AC (3)	90	11,13	7,8
	82	11,12	8,7
	94	11,11	8,10
Hb SC (4)	87	No flow	29,21
	82	40,45	16,18
	79	44,46	18,19
	76	23,25	15,16
Hb C-thal	61	18,16	12,12
Hb S-thal	76	10,11	7,8

* Range.

The filtration times for suspensions of either normal or Hb CC cells, equilibrated with propane, were each increased from 25 to 30%. The similar increase in viscosity and decrease in deformability for both normal and Hb CC cells, when equilibrated with propane, suggests that the abnormal viscosity of Hb CC did not result from a process similar to the sickling phenomenon with Hb S, such as *para-crystalline* formation or "molecular stacking" (25).

Studies in hypotonic media. The influence of the intracellular concentration of hemoglobin in Hb CC disease was examined because of the small MCV and high MCHC in Hb CC disease. The determination of viscosity at similar hematocrits was associated with a larger number of cells in the Hb CC samples due to the small MCV of Hb CC cells. Suspension of erythrocytes with Hb CC in hypotonic serum, decreasing the MCHC and increasing the MCV to normal, partially reversed the abnormal viscosity but did not reduce it to normal when the viscosities were compared at similar hematocrits and number of cells (Fig. 5). The filtration time was increased 50–80% for the Hb CC cells with the increase in MCV. In the normal

control blood, saline was added to the surum instead of water, which resulted in a decrease in the viscosity without a change in the filtration of the Hb AA cells. Similar findings were obtained with three different samples of Hb CC cells. Previous studies showed that the viscosity of normal cells was increased when the cell volume was increased in hypotonic media (16). These data indicate that the abnormal viscosity of erythrocytes with Hb CC was not entirely due to the high MCHC in these cells or the increased number of cells in the Hb CC samples when viscosity was compared with normal blood at similar hematocrits, but with different numbers of cells.

Pregnancy. Three patients with Hb CC were observed through pregnancy, each of which was uneventful, with the exception of anemia. The changes in each patient's hematocrit before and during pregnancy were: from 32 to 26%, 30 to 21% and from 31 to 27%. The respective changes in reticulocytes before and during pregnancies were: from 1.8 to 7.5%, 3.6 to 7.0%, and from 4.1

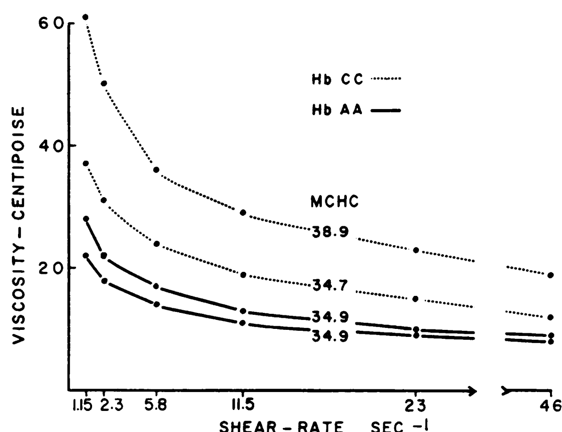

FIGURE 5 Blood viscosity in hypotonic media. Viscosity of Hb AA and Hb CC erythrocytes in serum and diluted serum at hematocrit 60.0 ± 0.5%, pH 7.4, and 37°C. The highest curve represents Hb CC cells in serum with the usually high MCHC, 38.9 g/100 ml. The next lower dotted curve represents Hb CC cells suspended in serum diluted with water to increase the cell volume and reduce the MCHC to 34.7 g/100 ml. The higher solid curve represents Hb AA cells in serum. The lower solid curve represents Hb AA cells in serum diluted with saline to the same degree that Hb CC cells were diluted with water, as a control on the dilution of serum proteins which decreases viscosity. In all suspensions the hematocrits were identical. There were similar numbers of cells in both the Hb AA samples and in the Hb CC sample with the lower viscosity.

to 12%, with each value representing at least two observations.

In view of the hematological changes that occur during pregnancy in patients with Hb CC and, in particular, because of the clinical problems, with Hb SC disease during pregnancy (1), the rheological properties of these cells were examined, both during pregnancy and by suspension of Hb SC and CC cells from nonpregnant patients in compatible serum or plasma obtained from pregnant patients near term. All cells (Hb AA, SC, and CC) showed an increased viscosity when suspended in either serum or plasma from 10 pregnant subjects. Fig. 6 shows the viscosity of Hb SC cells obtained during pregnancy and suspended in two different samples of plasma and serum. The viscosity in autologous plasma, obtained during pregnancy, was greater than the viscosity of the cells suspended in compatible plasma from a nonpregnant subject. Suspension of cells in serum obtained during pregnancy also resulted in a 15–25% increase in viscosity, compared with compatible serum from nonpregnant subjects. Results similar to those shown in Fig. 6 were observed with all cells studied (Hb AA, SC, and CC) in the serum and plasma of 10 pregnant subjects. There was a corresponding decrease in viscosity of cells from all of the pregnant patients when suspended in normal compatible serum from nonpregnant subjects. These studies indicate that during pregnancy there are changes in the serum in addition to the elevated fibrinogen in plasma (26) which increase blood viscosity. The increase in blood viscosity during pregnancy was not associated with any changes in the cells.

Because of the similarities in the elevation of the serum protein, transferrin, during pregnancy (27) and in the iron-deficient state, the viscosity of Hb AA, SC, and CC cells was observed in compatible serum from 10 iron-deficient patients. Suspensions of normal, Hb SC, or CC cells showed an increased viscosity (average 20%) when suspended in serum from iron-deficient patients with iron-binding capacities of 350–425 μg/100 ml compared with autologous "normal" serum. There was a corresponding decrease in viscosity of the abnormal iron-deficient cells when these were suspended in "normal" serum compared with autologous "iron deficient" serum. Dialysis of serum from both pregnant patients and iron-deficient patients

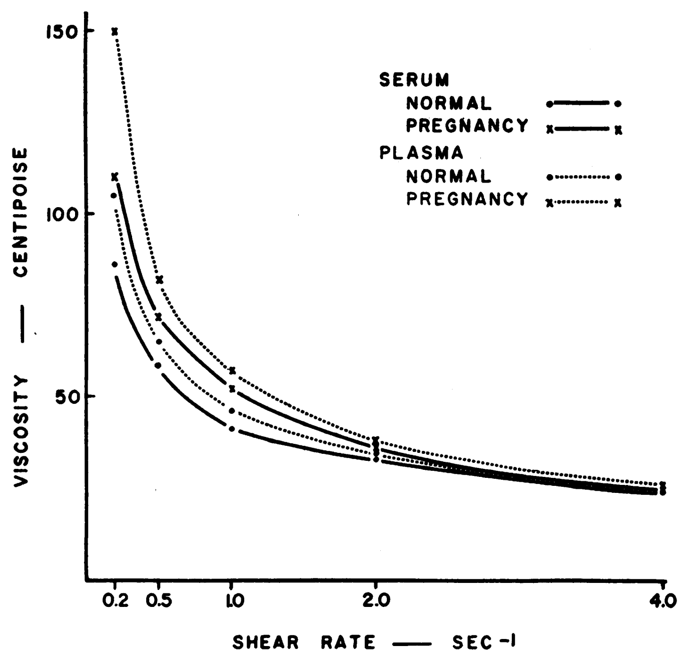

FIGURE 6 Blood viscosity at low shear rates. Viscosity determined in the GDM-viscometer for Hb SC erythrocytes in serum and plasma of pregnant patient (autologous) and from a non-pregnant patient at hematocrit 60.0 ± 0.2%, pH 7.4, and 37°C. The fibrinogen in normal plasma was 210 and 390 mg/100 ml in the plasma of the pregnant subject. The iron-binding capacity in the normal serum was 235 and 380 μg/100 ml in the serum from the pregnant subject.

did not alter the subsequent influence on the viscosity of cell suspensions. This property of serum from either pregnant or iron-deficient patients suggests that the iron-binding protein, transferrin, which is increased during pregnancy and with iron deficiency (27), alters the viscosity of erythrocyte suspensions. In contrast to the viscosity changes, there was no change in the filtration properties or deformability of Hb AA, SC, or CC cells in either serum or plasma from pregnant or iron-deficient subjects compared with autologous serum. These studies suggest that the increased viscosity in the abnormal serum was related to factors that altered cell-serum or cell-cell interactions and was not due to a change in the individual cell.

Cation studies. The Na and K content of erythrocytes with Hb C was found to be less than normal when calculated in relation to either hemoglobin content, cell volume, or cell water (Table III). Hb AC was associated with minimal changes in cation content. The possibility that low cation content in Hb CC disease was due to an abnormal permeability of the membrane was examined. Storage of cells in serum at hematocrit, 5% to reduce the effect of changes in concentration gradients during storage and at 5°C to prevent active transport, showed similar changes in Na and K for normal and Hb CC cells (Table IV). The changes in cation content must be evaluated in conjunction with the initial and final cation concentrations. These data indicate that at 5°C the permeability of the cell membrane to the passive transfer of Na and K was similar for both normal and Hb CC cells and that other factors result in the differences in cation content of the fresh Hb CC cells. Fresh cells from the heterozygous state, Hb SC, or AC, showed an intermediate decrease in cation content. Under identical storage conditions Hb SC cells showed greater changes in cation content than normal cells, indicating an increased permeability at 5°C to the passive transfer of cations. An increased permeability to cations has been previously described for cells with Hb SS (28).

TABLE III
Cation Concentration in Erythrocytes with Hb C

Subjects (No.)	Na/K per 350 g of Hb	Na/K per liter of RBC	Na/K per liter cell of H_2O
	meq(Mean ± SD)		
Normals (8)	11.1 ± 1.5/108 ± 2.5	11.5/112	
(4)			15.1/158
Hb CC (9)	5.4 ± 0.5/87 ± 2.0	5.9/95	
(4)			8.8/142
Hb AC (4)	9.6 ± 1.1/104 ± 3.1	9.6/104	
Hb SC (4)	9.6 ± 2.2/94 ± 4.0	10.4/102	
Hb C-thal (1)	6.3/100	6.4/102	

Table IV

Changes in Cation Content of Erythrocytes Stored for 30 hr at 5°C, pH 7.4, and hct 5%

Subject (Hb)	Na/K Control	30 hr	Balance
		meq/350 g of Hb	
AA	6.1/106	17.6/99	+11.5/−7.0
AA	8.4/106	19.0/98	+10.6/−8.0
AA	10.0/99	18.3/87	+ 8.3/−12
AA	8.5/105	20.1/94	+11.6/−11
AA	9.6/100	19.1/94	+ 9.5/−6
CC	4.2/87	16.9/76	+12.7/−11
CC	5.9/82	19.9/70	+14.0/−11
AC	7.9/99	17.5/87	+ 9.6/−12
SC	10.3/89	26.2/75	+15.9/−14
SC	14.4/82	24.4/62	+15.0/−20

Cell water. The amount of water in relation to cell volume was consistently smaller in Hb CC disease when compared with normal cells (Table V). The portion of cell water that participates in osmotic equilibrium, termed solvent water, was decreased to a greater extent than was cell water in Hb CC disease (Table V).

The solvent water content of Hb CC cells was calculated from the relative volume changes for both normal and Hb CC cells in similar hypotonic media and with a value of 80% for the solvent or osmotically available water in normal cells as determined by Savitz et al. (22). The per cent of volume change in 0.50% salt was based on MCV and MCHC data which agreed within ± 2%. The expected cell water in 0.50% salt was calculated from the volume change. The smaller volume change for Hb CC cells in hypotonic media suggests that less of the cell water participates in osmotic equilibrium. There was no change in cation content of the cells after suspension in 0.50% phosphate-buffered NaCl at pH 7.4. Bound water, which is the fraction of cell water that does not participate in osmotic equilibrium, is represented by the difference between total water and solvent water and is probably "bound" to the hemoglobin (29), although there is not complete agreement on the existence of bound or nonsolvent water (30). The smaller amount of solvent water in Hb CC cells as compared with normal cells, shown in Table VI, indicates that twice the amount of water was bound by Hb C, 29% of cell volume as compared with

Table V

Cell Water and Volume Change in 0.50% Salt Solution for Normal and Hb CC Cells at pH 7.4

Subjects	Cell water, % of volume	Equilibrated in 0.50% salt: % of volume increase	Cell water, % of new volume: Expected	Observed
Normal	70.9	45		
(Hb AA)	70.3	46		
	70.1	44	79.5	82.0
	71.0	46	80.2	82.0
Mean	70.6	45		
Hb CC	68.3	28		
	67.6	30		
	67.7	29	75.2	77.5
	66.0	34	76.9	77.2
Mean	67.4	30		

Hb A, 14% of cell volume. The relationship of cation and Hb concentrations in normal and Hb CC cells is also shown in Table VI. The difference in Hb concentration in normal and Hb CC cells was greater when calculated on the basis of cell water. The difference in cation concentration was less when calculated on the basis of cell water.

Table VI

Calculations of Cell Water, Hemoglobulin, Cation, and Chloride Concentrations in Four Normal Bloods and Hb CC Erythrocytes from Four Patients

Erythrocytes	Hb AA	Hb CC
Water, % of cell volume		
Total water	71	67
Solvent water	57	38
Bound water	14	29
Water bound to hemoglobin, *ml/g*	0.39	0.73
Hemoglobin, *g/100 ml*, cells per		
Cell volume	35.6	39.5
Cell water	50.1	58.9
Cations, Na plus K, *meq/liter*		
Cell volume	123	101
Cell water	173	151
Chloride ration, (concn–cell water)/(concn–serum water)	0.677	0.468

The ratio of chloride concentration [(concn in cell water)/(concn in serum water)] in normal cells was found to be similar to the data reported by Bromberg, Robin, and Jensen (31). In that study as well as these studies the pH was controlled by the pCO_2. The chloride content and ratio for Hb CC cells was less than for normal cells.

DISCUSSION

In Hb CC disease two separate or independent abnormalities involving cell water seem apparent: (*1*) the fraction of the cell volume that is water and, (*2*) the portion of the water that does not participate in osmotic equilibrium. The bound water is difficult to define chemically (29, 30, 32), but in this study it is defined as that portion of the cell water which does not participate in osmotic equilibrium, as measured by volume and water changes in hypotonic media.

In these studies the smaller volume change for Hb CC cells in hypotonic media, as compared to the volume change of normal cells in similar hypotonic media, indicates that a smaller fraction of the cell water in the Hb CC cell participates in osmotic equilibrium. Thus in Hb CC cells a larger fraction of the cell water is bound or nonsolvent water. The smaller chloride ratio and lower concentration of cations in Hb CC cells suggest that these ions are not uniformly distributed throughout the total water in the cell. When cation concentrations are calculated on the basis of solvent water, the concentration in Hb CC cells is greater than normal. When the chloride ratios were calculated on the basis of solvent water, the ratios for normal and Hb CC cells were equal. The distribution of chloride in the erythrocyte is not known. Recent studies of Cook (30) suggest that the chloride is distributed throughout the cell water and that 97% of the cell water was solvent water. The studies reported by Cook (30) were based on measurements of pH in whole blood and hemolysates, and are in contrast to estimates of solvent water found by volume change in hypotonic media, as shown by Cook (30) and Savitz et al. (22) as well as in this study.

The mechanism whereby Hb C binds more water than Hb A is not apparent. A difference in the molecular configuration of the Hb C molecule has been suggested on the basis of an increased number of titratable SH groups, eight per molecule of Hb C, as compared with six for Hb A (33). However, the similar effect of propane on the viscosity of cells with Hb AA, CC, or SS suggests that the hydrophobic sites on the different hemoglobin molecules are equally available. Other studies of molecular configuration such as x-ray diffraction or electron microscopy studies may prove fruitful.

The increased viscosity of Hb C could result from the greater amount of bound water altering the sol-gel state of the hemoglobin, or influencing intermolecular relationships. The exact state of Hb in normal cells is not known. The increased viscosity of Hb CC suggests that it is more like a gel than normal Hb. Charache, Conley, Waugh, Ugoretz, and Spurrell (12) suggested the increased viscosity of packed Hb CC cells resulted from a "precrystalline" state of the Hb related to alterations in electrostatic forces. However these authors were not able to demonstrate a difference in the viscosity of hemolysates of Hb AA and CC, which was found in the present study. Another possible mechanism that could alter viscosity would be a larger effective size of the Hb C molecule which would result from the extra bound water. The decreased amount of solvent or free water in the cell may partially account for the increased viscosity. However, increasing the solvent water by suspension in hypotonic media only partially reduced the abnormal viscosity and did not reduce the prolonged filtration time. Similar increases in cell volume and cell water of normal cells have been shown to increase the viscosity and filtration times (16). Thus, in Hb CC disease the high MCHC and decreased solvent water only partially account for the increased viscosity and decreased deformability of the cell. The greater viscosity of hemolysates with Hb C compared with Hb A at similar hemoglobin concentrations is further evidence that the abnormal viscosity is not entirely due to the high MCHC, but rather is a property of the Hb C molecule.

The smaller amount of water per liter of Hb CC cells does not entirely account for the low MCV in Hb CC disease. The amount of hemoglobin per cell is another factor that influences cell volume. In Hb CC disease the MCH or amount of hemoglobin per cell is less than normal. The low MCH in the homozygous state and the relatively greater amount of Hb A, as compared to Hb C in the

heterozygous state, has been interpreted as indicating a slower rate of synthesis of Hb C as compared to Hb A (2, 3). Thus, in Hb CC disease the small cell volume may result from both the smaller amount of hemoglobin per cell as well as less cell water, with relatively more water bound to hemoglobin and less as solvent water.

These studies do not provide an explanation for the abnormalities of cell water and electrolytes in the Hb CC erythrocyte. Hb C has a different charge than Hb A, due to the substitution of lysine for glutamic acid in the β-chain. This should result in a difference in net charge of + 2 meq/mmole of Hb for Hb C, and an alteration of the Donnan equilibrium. The Donnan effect is a difference in the concentration of small, diffusable ions on either side of a semipermeable membrane that results from the presence of charged, nondiffusable macro-ions on one side of the membrane (32). If this were the case in Hb CC cells and other nondiffusable anions were constant, the less negative charge of Hb C would be associated with a greater concentration of diffusable anions (Cl^-) within the cell. Although bicarbonate and organic phosphate compounds were not measured, the lower chloride ratio suggests that Donnon equilibrium was not significantly altered by the less negative charge of Hb C. The chloride ratio would be greater than normal in Hb CC cells if this were the case. A better understanding of cell water and the distribution of anions and cations within the cell may resolve these questions regarding the Hb CC erythrocyte.

The abnormal appearance and diminished osmotic fragility of Hb CC cells reflects a difference from normal in the relationship of volume and surface area. Target cells are an artifact of drying in a blood film. This phenomenon occurs when there is a relative increase in surface area of the membrane or decrease in cell volume. The determinations of the volume of cells at the time of osmotic lysis indicates that Hb CC cells lyse at a smaller volume than do normal cells and therefore they have less surface area. The decreased osmotic fragility, 50% lysis in a more hypotonic solution than for normal cells, is also related to the smaller amount of solvent water in the cell. The mean values suggest that the volume of the Hb CC cell is reduced to a greater extent than is the surface area of the membrane. The fact that the cells which appear as microspherocytes on the blood films do not lyse as microspherocytes in the osmotic fragility test is probably due to the abnormal hemoglobin-water relationship in these cells. The microspherocytes in Hb CC disease do not shown an increased osmotic fragility because the volume increase in hypotonic media is less than normal. The smaller mean surface area of the Hb CC cell may result from a mechanism similar to the process in iron deficiency anemia, presuming that the rate of hemoglobin synthesis is decreased in both conditions. Nevertheless, in Hb CC disease, the altered surface area to volume relationship appears to result from a minimal decrease in surface area and a marked decrease in volume, both factors which contribute to the abnormal appearance and decreased osmotic fragility of the rather heterogenous population of cells.

The decreased deformability or increased rigidity of the Hb CC erythrocyte is of greater clinical significance than the blood viscosity. The increased viscosity of the blood is the result of the abnormalities of the individual cell with its increased internal viscosity as well as the abnormal cell that influences the viscosity of a suspension of cells by altering cell-cell interactions in a laminar flow system. Patients with Hb CC disease do not show any particular clinical manifestations that could be related to an increased blood viscosity other than anemia. The anemia results in the blood being less viscous. The one area of the microcirculation where Hb CC erythrocytes appear abnormal is in the spleen. The lack of deformability of the Hb CC cell appears to correlate with the phenomenon of splenic sequestration.

Weiss (34) has demonstrated by electron microscopy studies of serial sections of the spleen that, the predominant circulation in the red pulp of the spleen is not by direct connections of arteriole to sinus to vein. In the areas of red pulp, arterioles terminated within the cords of reticuloendothelial cells and erythrocytes must pass between these cord cells and through a fenestrated basement membrane to enter the sinusoid. Some degree of deformability of the cell would be necessary for the cell to transverse this area of the microcirculation in the spleen. Previous studies (16) have demonstrated a marked loss of deformability of erythrocytes from patients with hereditary spherocytosis at a pH below 7.0, and

not at pH 7.4, which correlates with the process of splenic sequestration in that disease. The present study suggests that in Hb CC disease, splenic sequestration results from the lack of deformability of the cell, as indicated by the filtration data. In Hb SC disease, splenic sequestration is associated with changes in deformability, due to Hb C and the additional effect of the low pH of the spleen[4] that would enhance the sickling process in the spleen.

The significance of intracellular hemoglobin crystals in Hb CC disease is confusing in the literature. Various authors have quoted other investigators as having shown intracellular crystals in erythrocytes obtained from the spleen at the time of splenectomy. The original reports do not describe this phenomenon. Wheby, Thorup, and Leavell (10) examined dry smears made as splenic imprints and found no crystals in the erythrocytes from the spleen. Intracellular crystals in 1–2% of the cells have been seen on dried peripheral blood films taken after splenectomy (1, 9, 10). The formation of intracellular crystals in Hb CC cells during changes in the state of hydration of the cell, drying of blood films, or suspension in hypertonic media has suggested that Hb C is less soluble than Hb A (35, 11). The relationship of the low solubility and crystal formation to the unusual water distribution is not clear. The formation of Hb C crystals probably is related to a further change in either the abnormal cell water or hemoglobin-water relationship. In regard to the in vivo significance of crystals, intracellular crystals in fresh, wet preparations that have been kept at 37°C and not allowed to cool have not as yet been demonstrated. It should be noted that the abnormal rheological properties of Hb CC cells reported in this study were not associated with intracellular crystals of Hb C. The observations of Wheby et al. (10) fail to support the concept (11) that splenic sequestration results from the presence of intracellular crystals. The increased number of intracellular crystals seen on dried blood films following splenectomy suggests that crystal formation may be related to cell age. Further changes in the hydration of the hemoglobin molecule with aging could be related to increased crystal formation.

[4] To be published.

In Hb SC disease the presence of Hb C in conjunction with Hb S has been shown to potentiate the sickling process (36). Erythrocytes with Hb SC sickled at both higher oxygen tensions and pH as compared with cells with Hb SA (37, 38). The enhancement of sickling with Hb C has correlated with most of the clinical findings associated with Hb SC disease. In this study the increased viscosity and decreased deformability of the Hb SC cell, in the absence of sickling, correlates with the content of Hb C. In the heterozygous state the rheological properties were more abnormal with Hb SC than with Hb AC, which is consistent with the previous suggestion (36) of a specific or different type of interaction between Hb S and C, as compared to the interaction between Hb S and A or Hb C and A. The abnormal rheological properties of the Hb SC cell in the nonsickled state may be of equal importance to the sickling process in causing the clinical manifestations seen in Hb SC disease.

The significant increases in blood viscosity that occur during pregnancy may have a causal relationship to the increased hemolysis in Hb CC disease during pregnancy. In Hb SC disease the increased severity of the clinical manifestations, including the hemolytic process and vascular complications during pregnancy, may be related to the sickling process and the changes in viscosity. The changes in viscosity during pregnancy resulted from changes in the plasma proteins and were due in part to the increased fibrinogen (39) and, possibly, the increased transferrin. In Hb SC disease during pregnancy, the additional increase in viscosity related to changes in plasma proteins could result in significant changes in the microcirculation, particularly in the post capillary venules. In areas of low shear rates, or reduced rate of flow, the increased viscosity could lead to further decreases in flow and chemical changes to produce sickling. The sickling phenomenon would be an important contributing factor in patients with Hb SC disease during pregnancy, as patients with Hb CC during pregnancy develop only an increased degree of anemia.

ACKNOWLEDGMENT

This work was supported by Research Grant AM 02189 and Career Development Award GM 21856 from the National Institute of Arthritis and Metabolic Diseases, U. S. Public Health Service.

REFERENCES

1. Smith, E. W., and J. R. Krevans. 1959. Clinical manifestations of hemoglobin C disorders. *Bull. Johns Hopkins Hosp.* **104**: 17.
2. Thomas, E. D., A. C. Motulsky, and D. H. Walters. 1955. Homozygous hemoglobin C disease. *Am. J. Med.* **18**: 832.
3. Jensen, W. N., R. A. Schoefield, and R. Agner. 1957. Clinical and necropsy findings in hemoglobin C disease. *Blood.* **12**: 74.
4. Singer, K., A. Z. Chapman, S. R. Goldberg, H. M. Rubinstein, and S. A. Rosenblum. 1954. Studies on abnormal hemoglobins. IX. Pure (homozygous) hemoglobin C disease. *Blood.* **9**: 1023.
5. Ransone, J. W., and R. D. Lange. 1957. Homozygous hemoglobin C disease in a 79 year old man with gout. *Ann. Internal Med.* **46**: 420.
6. Dacie, J. V. 1960. The Hemolytic Anemias. Grune and Stratton, Inc., New York. Pt. 1.
7. Hunt, J. A., and V. M. Ingram. 1958. Allelomorphism and the chemical differences of the human hemoglobins A, S, and C. *Nature.* **181**: 1062.
8. Tanaka, K. R., and G. O. Clifford. 1958. Homozygous hemoglobin C disease: report of three cases. *Ann. Internal Med.* **49**: 30.
9. Diggs, L. W., A. P. Kraus, D. B. Morrison, and R. P. T. Rudnicki. 1954. Intraerythrocytic crystals in a white patient with hemoglobin C in the absence of other types of hemoglobin. *Blood.* **9**: 1172.
10. Wheby, M. S., O. H. Thorup, and B. S. Leavell. 1956. Homozygous hemoglobin C disease in siblings: further comment on intraerythrocytic crystals. *Blood.* **11**: 266.
11. Conley, C. L., and S. Charache. 1967. Mechanisms by which some abnormal hemoglobins produce clinical manifestations. *Seminars Hematol.* **4**: 53.
12. Charache, S., C. L. Conley, D. F. Waugh, R. J. Ugoretz, and J. R. Spurrell. 1967. Pathogenesis of hemolytic anemia in homozygous hemoglobin C disease. *J. Clin. Invest.* **46**: 1795.
13. Murphy, J. R. 1962. Erythrocyte metabolism. III. Relationship of energy metabolism and serum factors to the osmotic fragility following incubation. *J. Lab. Clin. Med.* **60**: 86.
14. Smithies, O. 1959. An improved procedure for starch-gel electrophoresis. *Biochem. J.* **71**: 585.
15. Huisman, T. H. J., and M. A. Dozy. 1962. Studies on the heterogeneity of hemoglobin IV. *J. Chromatog.* **7**: 180.
16. Murphy, J. R. 1967. The influence of pH and temperature on some physical properties of normal erythrocytes and erythrocytes from patients with hereditary spherocytosis. *J. Lab. Clin. Med.* **69**: 758.
17. Wells, R. E., Jr., R. Denton, and E. W. Merrill. 1961. Measurement of viscosity of biologic fluids by cone plate viscometer. *J. Lab. Clin. Med.* **57**: 646.
18. Merrill, E. W., E. R. Gilliland, G. Cokelet, H. Shin, A. Britten, and R. E. Wells, Jr. 1963. Rheology of human blood, near and at zero flow; effects of temperature and hematocrit level. *Biophys. J.* **3**: 199.
19. Jandl, J. H., R. L. Simmons, and W. B. Castle. 1961. Red cell filtration and the pathogenesis of certain hemolytic anemias. *Blood.* **18**: 133.
20. Nicolau, C. T., P. Teitel, M. Fotino, E. Butoianu, and S. Targar. 1964. Alterations of erythrocyte plasticity in blood diseases. *Sangre.* **9**: 282.
21. Murphy, J. R. 1963. Erythrocyte metabolism. V. Active cation transport and glycolysis. *J. Lab. Clin. Med.* **61**: 567.
22. Savitz, D., V. W. Sidel, and A. K. Solomon. 1964. Osmotic properties of human red cells. *J. Gen. Physiol.* **48**: 79.
23. Catlove, E., H. V. Trantham, and R. L. Browman. 1958. An instrument and method for automatic, rapid, accurate, and sensitive titration of chloride in biologic samples. *J. Lab. Clin. Med.* **51**: 461.
24. Ponder, E. 1948. Hemolysis and Related Phenomena. Grune and Stratton, Inc., New York. 85.
25. Murayama, M. 1964. A molecular mechanism of sickled erythrocyte formation. *Nature.* **202**: 258.
26. Gram, H. C. 1922. The results of a new method for determining the fibrin percentage in blood and plasma. *Acta Med. Scand.* **56**: 107.
27. Bothwell, T. H., and C. A. Finch. 1962. Iron Metabolism. Little, Brown and Co., Boston.
28. Tosteson, D. C. 1955. The effects of sickling on ion transport. II. The effect of sickling on sodium and cesium transport. *J. Gen. Physiol.* **39**: 55.
29. Drabkin, D. L. 1950. Spectrophotometric studies. XV. Hydration of macro-sized crystals of human hemoglobin, and osmotic concentrations in red cells. *J. Biol. Chem.* **185**: 231.
30. Cook, J. S. 1967. Nonsolvent water in human erythrocytes. *J. Gen. Physiol.* **50**: 1311
31. Bromberg, P. A., J. Theodore, E. D. Robin, and W. N. Jensen. 1965. Anion and hydrogen ion distribution in human blood. *J. Lab. Clin. Med.* **66**: 464.
32. Tanford, C. 1961. Physical Chemistry of Macromolecules. John Wiley and Sons, Inc., New York.
33. Murayama, M. 1958. Titratable sulfhydryl groups of hemoglobin C and fetal hemoglobin at 0° and 38°. *J. Biol. Chem.* **230**: 163.
34. Weiss, L. 1965. The structure of the normal spleen. *Seminars Hematol.* **2**: 205.
35. Kraus, A. P., and L. W. Diggs. 1956. In vitro crystallization of hemoglobin occurring in citrated blood from patients with hemoglobin C. *J. Lab. Clin. Med.* **47**: 700.
36. Griggs, R. C., and J. W. Harris. 1956. The biophysics of the variants of sickle cell disease. *Arch. Internal Med.* **97**: 315.
37. Allison, A. C. 1957. Properties of sickle-cell hemoglobin. *Biochem. J.* **65**: 212.
38. Charache, S., and C. L. Conley. 1964. Rate of sickling of red cells during deoxygenation of blood from persons with various sickling disorders. *Blood.* **24**: 25.
39. Wells, R. E., Jr., T. H. Gawronski, P. J. Cox, and R. D. Perera. 1964. Influence of fibrinogen on flow properties of erythrocyte suspensions. *Am. J. Physiol.* **207**: 1035.