Sickle-cell hemoglobin: Fall in osmotic pressure upon deoxygenation
(kinetic versus matrix osmosis/gelation/cellular dehydration and stiffening/molar and molal solute concentrations/erythrocyte)

ALAN R. HARGENS, LEMUEL J. BOWIE*, DEBORAH LENT, SYLVESTER CARREATHERS, RICHARD M. PETERS, H. T. HAMMEL, AND P. F. SCHOLANDER
Veterans Administration Medical Center and University of California, San Diego, California 92161; and Physiological Research Laboratory, Scripps Institution of Oceanography, University of California, San Diego, La Jolla, California 92038

Contributed by P. F. Scholander, April 3, 1980

ABSTRACT Macromolecules such as hemoglobin exert both kinetic and matrix effects on osmotic pressure. The kinetic osmotic pressure of sickle-cell hemoglobin is lost upon deoxygenation at physiological erythrocyte concentrations. The non-kinetic or matrix component of osmotic pressure remains relatively unchanged. Loss of thermal-osmotic activity during deoxygenation occurs throughout a hemoglobin concentration range between 2.5 and 35 g/100 ml. Deoxygenation of sickle-cell hemoglobin causes aggregation such that the matrix effect is unchanged but the kinetic (van’t Hoff) effect nearly vanishes. A loss of intracellular osmotic pressure during deoxygenation could dehydrate the erythrocyte sufficiently to promote more rapid sickle-cell hemoglobin aggregation. Subsequently, complete gelation of these aggregates could cause additional water loss and thrust the sickled cell into an irreversible cycle. The osmotic pressure of normal hemoglobin does not change appreciably during deoxygenation and is essentially the same as the osmotic pressure of oxygenated sickle-cell hemoglobin.

Sickling of deoxygenated erythrocytes containing hemoglobin S (Hb S) results from an aggregation of Hb S tetramers into a gel of long, multistranded polymers (1–5). The Hb S molecule differs from normal hemoglobin by a single amino acid substitution in each β-globin polypeptide chain (6). This turns the erythrocytes into long stiff convolutes. The major factors that promote high Hb S gelation are low oxygen tension and low pH (7–10). Distortion and stiffening of the cells, even though the erythrocytes are generally smaller than normal, give rise to many of the clinical manifestations of the sickle cell anemia.

Perutz and Mitchison have proposed that Hb S cell volume is lost during sickling (11). Studies aimed at determining experimentally whether the loss of cell volume by sickling is due exclusively to water loss have yielded conflicting results (12–15). Because the loss of water by cells containing Hb S would lead by itself to higher intracellular hemoglobin concentrations, both speed and extent of aggregation would increase (16) as these cells are exposed to hypoxia. A unified reevaluation of the osmotic mechanism (17) implied to us that the osmotic behavior of Hb S may significantly change during sickling. Whether the state of oxygenation per se would result in a thermal-osmotic effect or a matrix-swelling effect on various hemoglobins seemed unclear.

METHODS

The osmotic effect on Hb S was measured after oxygenation and deoxygenation in a wide range of concentrations obtained by a simple dilution procedure, going from 35 to 2.5 g of hemoglobin per 100 ml. Similarly, the osmotic behavior of normal hemoglobin (Hb A) was studied in its oxygenated and deoxygenated states in the same range of concentration.

Venous blood was drawn from subjects homozygous for Hb S or Hb A. The anticoagulant-treated samples were centrifuged at 1300 × g and washed three times with 0.9% NaCl. The packed cells were lysed in an equal volume of distilled water, followed by extraction of membrane lipids with an equal volume of chloroform. Cell debris were removed by centrifugation at 1300 × g for 15 min, and the hemolysate was carefully removed by aspiration. The resulting hemoglobin solution was dialyzed and concentrated, using a membrane with an exclusion limit of 25,000 daltons. A sample was converted to cyano-methemoglobin and the concentration was determined by absorbance at 450 nm, using a Cary 118 C spectrophotometer. These purified hemoglobin solutions were concentrated to slightly above 35 g/100 ml and subsequently diluted to produce a series of hemoglobin concentrations from 35 to 2.5 g/100 ml.

Each hemoglobin sample was placed in a glass tonometer and equilibrated at 22° C with humidified gas [pO2 was 100 mm Hg and 0 mm Hg for oxy and deoxy forms, respectively; the remaining gas was N2 (1 mm Hg = 133 Pa)]. During each measurement of osmotic pressure, the respective gases were continuously passed over the sample.

Osmotic pressure in the purified hemoglobin solution was measured in a simple Plexiglas colloid osmometer (18), which combined membrane rigidity with accuracy to within 1%. A dialysis membrane with a molecular-weight retention of 10,000 was stretched over a lens paper spacer and rubber support to maintain membrane rigidity so that osmotic pressures up to 500 mm Hg were measurable. Osmotic equilibrium was obtained by counteracting the osmotic pressure with an equal and opposite hydrostatic pressure applied by a syringe-type manometer (19).

After the osmotic pressure of a 35 g/100 ml oxy-HbS solution had been measured, the sample was deoxygenated by tonometry and returned to the osmometer for measurement of deoxy-HbS osmotic pressure. The deoxygenated sample was noticeably more viscous in the upper concentration range, and the Hb S gel was therefore carefully spread over the entire membrane surface. After equilibrium was obtained, the Hb S sample was oxygenated a second time in the tonometer and the osmotic pressure was rechecked. A 2-ml sample was then diluted slightly by addition of 0.154 ml of buffered saline to obtain a lower Hb S concentration (32.5 g/100 ml) for comparing osmotic pressures of oxygenated and deoxygenated states again. Subsequently, the sample was diluted stepwise down to 2.5 g/100 ml and Hb S concentration was determined spectrophotometrically after each dilution. Osmotic pressures for

Abbreviations: Hb S, sickle-cell hemoglobin; Hb A, normal hemoglobin.
* Present address: Clinical Biochemistry, Evanston Hospital, 2650 Ridge Avenue, Evanston, IL 60201.
oxy-Hb S and deoxy-Hb S were determined at each concentration. In a similar manner, osmotic pressures for oxy-Hb A and deoxy-Hb A were measured in a concentration range of 35 to 2.5 g/100 ml.

RESULTS

The osmotic pressure of normal hemoglobin was the same for oxygenated and deoxygenated hemoglobin at all concentrations (Fig. 1). Furthermore, it is clear that the osmotic pressures of normal oxy- and deoxyhemoglobin molecules increase more rapidly than an increase in their molar concentrations, the line labeled "van't Hoff contribution" in Fig. 1. This deviation of osmotic pressure from the van’t Hoff line is characteristic of large molecules.

The osmotic pressure of all oxy-Hb S samples (Fig. 2) was not distinguishable from the osmotic pressure of either oxy-Hb A or deoxy-Hb A at the same concentration. In other words, the curve for oxy-Hb S in Fig. 2 will superimpose on the curve for oxy-Hb A and deoxy-Hb A in Fig. 1. On the other hand, the osmotic pressure of all deoxy-Hb S samples was markedly less than for the the oxy-Hb S samples (Fig. 2). At 35 g/100 ml, a concentration within physiological limits, osmotic pressure of deoxy-Hb S was approximately 100 mm Hg less than that for oxy-Hb S. At the lowest concentrations, the osmotic pressure of deoxy-Hb S was not measureably above zero.

DISCUSSION

Small molecules dissolved in water affect the osmotic pressure of the solution an amount that is proportional to the absolute temperature and the molar concentration up to at least 1 or 2 mol/kg of water. This linear dependence on temperature and number of molecules per unit volume clearly demonstrates that the kinetic energy (thermal motion) of the solute molecules alone determines the osmotic pressure of the solution. Large molecules such as hemoglobin, in addition to their kinetic effect on osmosis, exhibit a nonkinetic effect, which may be characterized as a matrix effect that exerts osmotic pressure even at low concentrations. The molar concentration of 35 g of Hb A per 100 ml is only about 0.008 mol/kg of water, whereas the volume of the hemoglobin is more than a third the volume of the solution.

When deoxy-Hb S molecules aggregate, we may anticipate little or no change in their total volume and, therefore, little or no change in their nonkinetic contribution to osmotic pressure. At the same time, we may anticipate that their kinetic contribution will be greatly diminished. According to this interpretation, the curve for oxy-Hb S in Fig. 2 minus the curve for deoxy-Hb S should equal RT(molar concentration), the kinetic
effect. The broken line in Fig. 2 is the regression line for the difference between the osmotic pressures of the oxy-Hb S and deoxy-Hb S solutions as a function of concentration. Its slope is a little more than the van't Hoff line, $RT$ (molar concentration), but significantly less than $RT$ (molal concentration). We conclude from this that: (i) the large molecules of oxy-Hb S exert a kinetic and matrix effect on the osmotic pressure and (ii) assuming that loss of oxygen causes the Hb S molecules to aggregate or gel such that their distribution and matrix effect is unaltered, then their kinetic effect is greatly diminished.

Osmotic events during deoxygenation of Hb S cells may play an important role in the development of decreased volume and greater rigidity of erythrocytes in Hb S patients. In addition, a process of erythrocyte dehydration may lead to the generation of irreversibly sickled cells. Osmotic pressure measurements may therefore prove useful for monitoring events occurring very early in the Hb S aggregation process or at low concentrations of Hb S.

We thank Drs. F. N. White, A. B. Hastings, R. C. Blantz, H. A. Itano, H. M. Ranney, and V. S. Sharma for helpful discussions, and B. J. Sverdrup for assistance in manuscript preparation. This research was supported by the Veterans Administration and by U.S. Public Health Service Grants GM-24901 and AM-25501 and Research Career Development Award AM-00602 to A.R.H.