EFFECT OF POLYAMINES ON THE ELECTROKINETIC PROPERTIES OF RED BLOOD CELLS*

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Received February 27,1976

SUMMARY

At the physiological pH 7.4, the zeta potential of the normal red blood cell in 1.5% glycine buffer was found to be -52 mv, whereas that of sickling erythrocytes is -45 mv. Addition of spermidine to normal red blood cells reduced the zeta potential by approximately 20 mv. In sickling red blood cells, where the polyamine content is determined to be 5 to 6 times greater than in the normal erythrocyte, addition of spermidine reduced the zeta potential by only 5 mv, indicating that little more polyamine binding occurs. The polyamine content of whole blood taken from 24 patients having sickle cell anemia was found to be more than ten times that of whole blood from normal donors. Binding of polyamines to the normal red blood cell was analyzed from the surface charge potential variation as a function of polyamine concentration and the apparent binding constant determined to be 130 dl/g. The difference in the electrokinetic properties of normal and sickling red blood cells in this system may be attributed, in part, to a variation in the polyamine content of the two types of erythrocytes.

INTRODUCTION

Information about the surface alteration and deformation of the human red blood cell may be obtained by examining the electrokinetic behavior of its electrical double layer (1-8). It has been suggested that N-acetyl neuraminic acids, which possess a very low pK value in crystalline form, may be responsible for the electrokinetic properties of the red blood cell surface (9-11).

The Gouy-Chapman equation (3,12-14) for surface charge density has been used to calculate the electrokinetic charge density distribution of the red blood cell (3). Heard and Seaman (6) found that these values fit the Langmuir binding isotherm.

It is known that the human erythrocyte possesses surface receptors capable of recognizing and binding a variety of external chemical substances. Glycophorin (15,16), the major glycoprotein, carries specificity for a number of blood group substances, although its exact function is not yet known.

*This work was supported by National Science Foundation Grant BMS 71-00850-A03 and, in part, by General Research Support, College of Medicine, University of Florida, and the C. F. Foundation.

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The fluid mosaic model of membrane structure (17), in which the matrix of the membrane is a bilayer of phospholipids, is generally accepted as a description of the molecular organization of most functional membranes. In the case of erythrocyte membranes, the evidence indicates that the proteins and phospholipids are asymmetrically distributed in the two halves of the bilayer (18,19).

In examining human erythrocytes by fluorescence labeling, Larsen (29) has demonstrated that the interaction of polyanions with membrane-bound polycations leads to the formation of polar bonds and a subsequent reduction in the repulsive potential, or negative surface charge, of the red blood cells.

Sheetz and Singer (20) propose that certain compounds which induce shape changes in the intact erythrocyte do so by intercalating their hydrophobic ends into the non-polar interior of the lipid portions of the membrane, while their polar or ionic ends are exposed at the surface. Hence, the integral proteins of the red blood cell membrane would not be involved in the binding of these components or the cell shape changes that result.

Polyamine analysis in bone marrow samples of leukemic and non-leukemic conditions, using the amino acid analyzer, reveal increased concentrations of putrescine, spermidine, and spermine in leukemic cells (21-23), and elevation and alteration of the polyamine content of blood in the inherited disorder, cystic fibrosis (22,23), which is identified by characteristic dysfunction of the exocrine system.

To date, no findings have been reported on the polyamine content of sickling red blood cells. Little is known about the possible role of polyamines in the deformation and rigidity of the red blood cell *in vitro* or *in vivo*.

In this communication, we report a notable elevation in the polyamine content of sickling red blood cells when compared to that of the normal erythrocyte. Measurement of the zeta potentials of the two types of erythrocytes reveals a charge variation in the red blood cell membrane which may account in part for the observed variation in polyamine binding.

MATERIALS AND METHODS

Isolation of Red Blood Cells. Ten to fifteen milliliters each of whole blood from donors with normal and homozygous sickling hemoglobin were collected in Heparin tubes and centrifuged at 900 r.p.m. in an HN-S centrifuge (International, a division of Damon) for 20 minutes prior to decanting the serum and leukocytes. The cells were washed eight times in 10 ml of isotonic 0.9% NaCl solution and centrifuged for 20 minutes at 1000 r.p.m. after each washing. A greater degree of adhesion was observed for the sickling red blood cell in solution during this washing procedure (24). All subsequent procedural operations were completed during a two-day period. Prolonged standing for greater lengths of time at 4°C resulted in a leaching of hemoglobin from the cells. Hematocrit values for red blood cells from sickle cell anemia patients were 25-35 as compared with 40-45 for erythrocytes from normal donors.

Electrophoretic Mobility of Red Blood Cells. The electrophoretic mobility of the red blood cell is measured in 1.5% glycine buffer as a function of pH with a Riddick Zeta Meter (Zeta Meter, Inc., New York). The electrophoretic mobility was computed from the current intensity, dimensions of the cell, and the specific conductance of the solution using the Helmholtz-Smoluchowski equation (4).

The zeta potential was also measured as a function of concentration of polyamine and $CaCl_2$. The concentration of red blood cells in suspension was diluted to an equivalent to 1×10^{-4} M/heme for each sample, using the ratio of molar extinction at 576 to 541 nm of 1.066.

Tryptic Digestion. Whole white ghost erythrocyte membranes isolated from red blood cells (25) were digested with trypsin and two-dimensional peptide mapping was performed as described elsewhere (26,27).

Analysis of Polyamine. One ml of heparinized blood or washed red blood cells was extracted with an equal volume of 10% sulfosalicylic acid. The mixture was stirred for a few minutes and then centrifuged at 8000 r.p.m. for 20 minutes. Extraction was repeated twice. The supernatant was lyophilized and resuspended in 0.5 ml of 0.2 N sodium citrate buffer, pH 2.2, and then filtered through a millipore filtration apparatus. Polyamine content was determined using the Durrum D-500 high-pressure chromatographic analyzer coupled with a digital data PDP/M coupler.

RESULTS

The electrophoretic properties of normal and sickling red blood cells, examined in 1.5% glycine buffer as a function of pH, show distinct differences in the zeta potentials of the two erythrocytes, as shown in Figure 1. At the physiological pH 7.4, the zeta potential of normal red blood cells is about -52 mv, whereas that of sickling erythrocytes is -45 mv.

The electrophoretic mobilities of normal red blood cells at pH 7.4 were found to be -1.2 $\mu m\ sec^{-1}v^{-1}$ cm in 5% sucrose and -2.0 $\mu m\ sec^{-1}v^{-1}$ cm in M/15 phosphate buffer, with a standard deviation of ±0.61. Glycine buffer was found to be best suited for measuring the electrophoretic properties of the red blood cell (30). The isoelectric point of the normal red blood cell in glycine buffer is 4.8. This is in contrast to isoelectric points ranging from 1.7 to 4.5 for buffers such as sucrose, phosphate, sorbitol, or citrate (6,8). In our experiments, the pI of the red blood cell in 5% sorbitol and sucrose are 5.8 and 6.1, respectively. It also is obvious from our measurements that the increase in zeta potential as a function of pH is slow and gradual for red blood cells in sucrose or sorbitol buffer, with a maximum zeta potential of -38 mv (2.8 $\mu m\ sec^{-1}v^{-1}$ cm) at pH 10.2 and -45 mv (3.4 $\mu m\ sec^{-1}v^{-1}$ cm) at pH 10.0.

Analysis of the tryptic digest of whole ghost erythrocytes reveals differences in the polyamine content of normal and sickling red blood cells. Addition of spermidine or spermine at concentrations up to 5.0×10^{-3} M to the normal red blood cells reduces the zeta potential by approximately 20 mv. The reduction for sickling erythrocytes is only 5 mv, as shown in Figure 2. The effect of spermidine, as may be seen in this figure, is far greater than that of $CaCl_2$, although both would appear to undergo biphasic or sigmoidal binding with

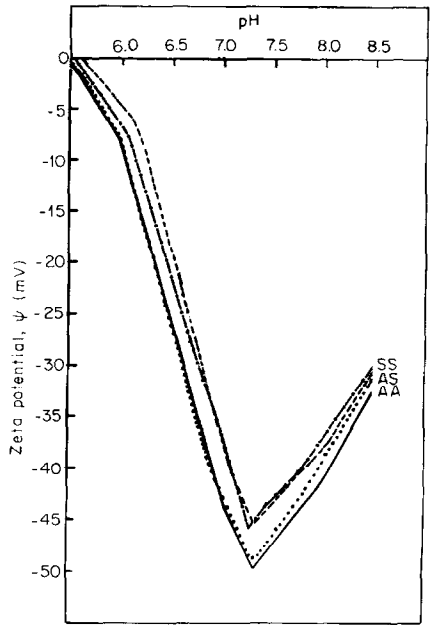

FIGURE 1

Comparison of the zeta potential as a function of pH of red blood cells from normal donors and persons with various hemoglobin disorders in 1.5% glycine buffer.

AA - Normal red blood cells.
AS - Heterozygous sickling red blood cells.
SS - Homozygous sickling red blood cells; dashed line shows the degree of experimental variation.

Each pH value represents the average of ten measurements, with a standard deviation of 5%. Measurements were taken within two days after blood samples were drawn for a number of experiments over a one-year period.

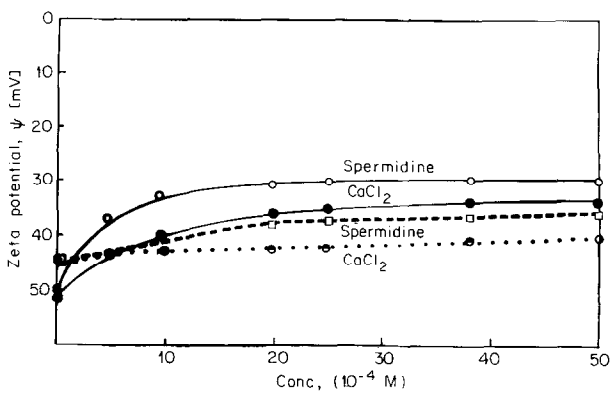

FIGURE 2

The zeta potentials of normal (● or 0) and sickling (···· or ----) red blood cells as a function of ligand concentration in the presence of spermidine and $CaCl_2$. Cells were incubated with ligand at 37°C for two hours. Zeta potential is measured in 1.5% glycine buffer, pH 7.4.

TABLE I

POLYAMINE CONTENT OF LEUKOCYTES AND ERYTHROCYTES (nmoles/10^9 cell)

	Leukocytes			Erythrocytes		
	Putrescine	Spermidine	Spermine	Putrescine	Spermidine	Spermine
Normal (N = 9)	2.99 ± 1.50	15.33 ± 0.5	35.95 ± 0.91	0.007~	1.39 ± 0.46	0.9 ± 0.27
Sickle Cell (N = 2)	Trace	238 ± 11.9	322.5 ± 12.1	0.01~	6.81 ± 0.4	6.95 ± 0.3

N represents the number of donors of blood samples.

the red blood cell. It may be inferred from these results that little or no further polyamine binding occurs after the addition of spermidine to sickling erythrocytes.

In sickling red blood cells, the polyamine content by high-pressure chromatographic analysis is found to be five or six times greater than that of normal erythrocytes, as shown in tabular form below.

In the leukocytes, the spermine content is ten times greater in blood from sickle cell anemia patients than that from normal donors. For spermidine, this difference is 15-fold, as shown in Table I.

The polyamine content of whole blood taken from 24 patients having sickle cell anemia has been found to be approximately ten times greater than that of whole blood taken from normal donors. For spermine, the difference is three-fold, as shown in Table II.

DISCUSSION

From measurements of the zeta potentials of normal and sickling erythrocytes, we may conclude that the surface charge variation is a manifestation of basic differences in the composition of the cell membrane of the two erythrocytes.

Assuming that the zeta potential of the red blood cell is -52 mv at pH 7.4, then the electrical double layer field would be 5.2×10^5 v/cm, provided that the thickness of the electrical double layer is approximately 10 Å. The total surface charge densities of normal and sickling red blood cells, calculated from the Gouy-Chapman equation (3,13) for electrolytes as a function of the ionic layer at a distance of 5 Å toward the surface from the shearing plane at the physiological pH for univalent electrolytes, are calculated to be 58.41×10^3 stat coulombs/cm^2 and 36.16×10^3 stat coulombs/cm^2, respectively (28).

TABLE II

WHOLE BLOOD POLYAMINE CONTENT (nmoles/ml)

	Sickle Blood* (N = 24)	Normal Blood (N = 4)
Putrescine	0.42 ± 0.05	Trace
Cadaverine	-----	-----
Spermidine	35.97 ± 17.9	3.87 ± 1.29
Spermine	13.52 ± 5.41	4.01 ± 1.37

*Range: Putrescine, 0.1~1.14 nmoles/ml; Spermidine, 14~78.0 nmoles/ml; Spermine, 3.0~44 nmoles/ml.

Our calculations show a 30% reduction in the surface charge density of sickling red blood cells over that of the normal erythrocytes. This compares to a reduction in zeta potential of about 13%, sickling cells having a zeta potential of -45 mv.

Binding of polyamines to the normal red blood cell was analyzed using the Gouy-Chapman equation for surface charge density variation as a function of polyamine concentration. The apparent binding constant was determined to be 130 dl/g, based on the data shown in Figure 2.

It is reasonable to conclude that the differences in the electrokinetic properties of normal and sickling red blood cells which we have observed may be attributed in part to a variation in the polyamine content of the two types of erythrocytes.

Since the erythrocytes do not have a mechanism for the synthesis of polyamines, we hypothesize, based on the data in Tables I and II, that quantities of spermine and spermidine are carried by the leukocytes, with an equilibration of polyamines between leukocytes and erythrocytes taking place during circulation.

Leukocytes $\rightleftharpoons$ Serum $\rightleftharpoons$ RBC

Binding to the sickling red blood cell is most pronounced, possibly by intercalation of the polyamines into the erythrocyte membrane, as proposed by Sheetz and Singer for a number of amphipathic compounds.

To date, despite wide discussion in the literature, there has been no direct experimental evidence that the sickling of the human erythrocyte is a result of some alteration in the hemoglobin molecule itself. Based on our findings, we theorize that the aggregation of sickle cell hemoglobin may in fact be membrane-facilitated, with the erythrocyte membrane itself playing a role in the sickling process.

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