Influence of the Ionic Composition of Fluid Medium on Red Cell Aggregation

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ABSTRACT The effects of ionic strength and cationic valency of the fluid medium on the surface potential and dextran-induced aggregation of red blood cells (RBC's) were investigated. The zeta potential was calculated from cell mobility in a microelectrophoresis apparatus; the degree of aggregation of normal and neuraminidase-treated RBC's in dextrans (Dx 40 and Dx 80) was quantified by microscopic observation, measurement of erythrocyte sedimentation rate, and determination of low-shear viscosity. A decrease in ionic strength caused a reduction in aggregation of normal RBC's in dextrans, but had no effect on the aggregation of neuraminidase-treated RBC's. These findings reflect an increase in electrostatic repulsive force between normal RBC's by the reduction in ionic strength due to (a) a decrease in the screening of surface charge by counter-ions and (b) an increase in the thickness of the electric double layer. Divalent cations (Ca++, Mg++, and Ba++) increased aggregation of normal RBC's in dextrans, but had no effect on the aggregation of neuraminidase-treated RBC's. These effects of the divalent cations are attributable to a decrease in surface potential of normal RBC's and a shrinkage of the electric double layer. It is concluded that the surface charge of RBC's plays a significant role in cell-to-cell interactions.

INTRODUCTION

In the accompanying paper (1), it has been shown that the negative surface charge of human red blood cells (RBC's) generates an electrostatic repulsive force between adjacent cell surfaces in aggregates formed by macromolecular bridging. According to the theory of electric double layer (2), electrostatic repulsion between parallel surfaces is determined by the surface potential and the potential profile in the double layer, both of which are in turn functions of the ionic composition in the fluid medium. Thus, a decrease in ionic strength, by reducing the counter-ion concentration, leads to (a) an increase in the effective surface potential and (b) a decrease in the slope of the potential drop in the diffuse layer. Both of these effects would increase the electrostatic repulsive force between cells. We have proposed that red cell aggrega-
tion by macromolecules results when the macromolecular bridging force can overcome the electrostatic repulsive force (3). According to this model of RBC aggregation, therefore, a reduction in ionic strength should decrease RBC aggregation. An increase in cationic valency should have effects opposite to those of a decrease in ionic strength. The present experiments were designed to study the influence of ionic strength and ionic species on RBC surface potential and dextran-induced RBC aggregation.

MATERIALS AND METHODS

1. Electrophoretic Mobility Experiments

The electrophoretic mobility of suspensions of human RBC's (hematocrit 0.1%) was measured in a cylindrical microelectrophoresis apparatus as described in the preceding paper (1). The zeta potential ($\xi$ in millivolts) was calculated as:

$$\xi = \frac{360\pi \eta u}{D},$$

where $\eta$ is the viscosity (in centipoise) and $D$ is the dielectric constant of the fluid medium, respectively, and $u$ is the electrophoretic mobility (in $\mu m \cdot s^{-1} / V \cdot cm^{-1}$). Two series of experiments were conducted to investigate the influences of ionic strength and cationic species of the suspending medium on the electrophoretic mobility of normal RBC's.

A. IONIC STRENGTH-MOBILITY SERIES In this series, 0.15 M salt solutions (NaCl or MgSO$_4$) were mixed with 0.3 M sucrose solution in various ratios. The pH of each solution was adjusted to 7.4 ± 0.1 with 0.3 M tris(hydroxymethyl)aminomethane (Tris). Since very low ionic environments may cause instability of the electrophoretic behavior of RBC's (4, 5), salt concentrations below 5 mM were not included in the present study.

B. CATIONIC SPECIES-MOBILITY SERIES Normal RBC's were suspended in 0.15 M salt solutions containing NaCl and 2.5–10 mM of KCl, MgCl$_2$, MgSO$_4$, CaCl$_2$, BaCl$_2$, or LaCl$_3$. The solutions were prepared by replacing a portion of the NaCl in saline (0.15 M NaCl) with an equimolar amount of the other salts. The pH of each solution was adjusted to 7.4 ± 0.1 with 0.3 M Tris.

Electrophoretic measurements were made both in albumin-free solutions and in solutions containing 0.5 g/100 ml human serum albumin, and no difference was found.

2. RBC Aggregation Experiments

These studies were performed on normal RBC's and neuraminidase-treated RBC's. RBC aggregation by Dextran 80 and Dextran 40 was quantified with the use of three methods: microscopic aggregation index, erythrocyte sedimentation rate, and low-shear viscosity of the suspension. These methods are described in the accompanying paper (1). Two series of experiments were performed. In one series the ionic strength
of the suspending medium was varied, and in the other series various amounts of
divalent cations were used to replace sodium.

A. IONIC STRENGTH-AGGREGATION SERIES In this series saline (0.15 M NaCl)
was mixed with 0.3 M sucrose solution in ratios of 1:0, 2:1, and 1:2 to obtain iso-
osmotic solutions with ionic strengths (I) of 150, 100, and 50 mM, respectively.
Dextran 80 was dissolved in these three solutions to yield concentrations up to 10
g/100 ml.

B. CATION SPECIES-AGGREGATION SERIES Various amounts of divalent cation
salts (CaCl₂, MgCl₂, and BaCl₂) were used to replace an equimolar amount of
NaCl. Dextran 40 or Dextran 80 was dissolved in these solutions to give macro-
molecular concentrations up to 10 g/100 ml.

In both series of experiments, each solution contained 0.5 g/100 ml human serum
albumin to prevent crenation and hemolysis of RBC's (6). The pH of each solution
was adjusted to 7.4 ± 0.1 with 0.3 M Tris.

![Figure 1](image-url)

**Figure 1.** Effects of varying the concentration of salts on electrophoretic mobility (A)
and zeta potential (B) of RBC's. The osmolality is maintained at 300 mosmol with
addition of sucrose.
RESULTS

1. Ionic Strength of the Suspending Medium

A. EFFECT OF IONIC STRENGTH ON RBC SURFACE POTENTIAL The electrophoretic mobilities of normal RBC's in iso-osmotic NaCl-sucrose and MgSO₄-sucrose solutions at various ionic strengths are shown in Fig. 1 A. High ionic concentrations decreased the electrophoretic mobility of RBC's, and this decrease was greater with MgSO₄ than NaCl on an equimolar basis. With the use of dielectric increments of $-11 \text{ mol}^{-1}$ for NaCl and $-30 \text{ mol}^{-1}$ for MgSO₄ (7) and the viscosity values determined in the present experiments (Table I), Eq. 1 can be used to calculate the zeta potential from RBC

| Viscosities of NaCl and MgSO₄ Solutions of Various Ionic Strengths (Total Osmolality = 300 MOSMOL Balanced by Sucrose) at 25°C |
|---|
| Salt concn | Viscosity |
| NaCl | MgSO₄ |
| mM | cP | cP |
| 0 | 1.01 | 1.01 |
| 5 | 1.01 | 1.01 |
| 10 | 1.00 | 1.01 |
| 20 | 1.00 | 1.00 |
| 30 | 0.99 | 1.00 |
| 50 | 0.98 | 1.00 |
| 75 | 0.96 | 0.99 |
| 100 | 0.94 | 0.98 |
| 125 | 0.92 | 0.98 |
| 150 | 0.91 | 0.97 |

Figure 2. Effects of ionic strength (I) on aggregation of normal RBC's in NaCl solution plus Dextran 80 (total osmolality = 300 mosmol, balanced by sucrose). The figure gives the relation between microscopic aggregation index (MAI) and dextran concentration.
mobility data. As shown in Fig. 1 B, an increase in salt concentration decreased the zeta potential.

**B. EFFECT OF IONIC STRENGTH ON RBC AGGREGATION** Dextran 80 in saline ($I = 150$ mM) caused considerable aggregation of normal RBC's with an optimum concentration between 3 and 4 g/100 ml (Fig. 2). A decrease in ionic strength of the solution resulted in a reduction of the effectiveness of Dextran 80 in causing RBC aggregation. At $I = 100$ mM, Dextran 80 caused only slight RBC aggregation with the optimum concentration shifted to between 2 and 3 g/100 ml. When the ionic strength was further reduced to

**Figure 3.** Effects of ionic strength ($I$) on sedimentation curves of normal RBC's suspended in NaCl solutions containing 3 g/100 ml Dextran 80. Total osmolality = 300 mosmol, balanced by sucrose. Hematocrit = 45%.

**Figure 4.** Effects of ionic strength ($I$) on suspension viscosities of normal RBC's in NaCl solutions containing 3 g/100 ml Dextran 80. Total osmolality = 300 mosmol, balanced by sucrose. Hematocrit = 45%.
I = 50 mM, Dextran 80 failed to cause any RBC aggregation. The effects of varying ionic strengths on RBC aggregation by Dextran 80 as demonstrated by the sedimentation curves (Fig. 3) and the viscometric behavior of the RBC suspensions (Fig. 4) were in agreement with the direct microscopic findings.

In contrast to the RBC's with normal surface charge, the aggregation of neuraminidase-treated RBC's by Dextran 80 was not affected by variations in the ionic strength of the solution (Fig. 5). Results on sedimentation rate and viscosity measurement also indicated that a reduction in ionic strength had no detectable effect on dextran-induced aggregation of neuraminidase-treated RBC's.

![Figure 5. Effects of ionic strength (I) on aggregation of neuraminidase-treated RBC's in NaCl solution plus Dextran 80 (total osmolality = 300 mosmol, balanced by sucrose).](image)

2. Cation Species of the Suspending Medium

A. Effect of Cation Species on RBC Surface Potential. The electrophoretic mobility and the zeta potential of normal RBC's were determined in saline in which 0–10 mM of various salts were used to replace an equimolar amount of NaCl (Fig. 6). Since the concentration of the added salt was not more than 10 mM, the viscosity and the dielectric constant of each solution used for the calculation of the zeta potential (Eq. 1) were assumed to be the same as those of saline. The results indicate that divalent cations were more effective than monovalent ones in reducing the electrophoretic mobility and the zeta potential. On an equimolar basis, there was no difference between the effects of K+ and Na+. For divalent cations, there was also no detectable difference among Mg++, Ca++, and Ba++ with the concentrations used.1 Variations of anions (e.g., comparison of MgCl₂ and MgSO₄) did not affect

1 Although no significant difference was found among Ba++, Ca++, and Mg++ in their effects on electrophoretic mobility and cell aggregation with concentrations up to 10 mM, a gradation of these effects in the order of Ba++ > Ca++ > Mg++ was obtained at a concentration of 50 mM.
the electric properties of RBC's. The trivalent cation La+++ caused marked reduction and even reversal of the electrophoretic mobility and the zeta potential of RBC's. RBC's aggregated to form clumps of irregular shape during electrophoretic measurement when the LaCl₃ concentration was raised to 2.5 mM or higher.

B. EFFECT OF CATION SPECIES ON RBC AGGREGATION In saline without divalent cations, normal RBC's showed slight aggregation in Dextran 40 and considerable aggregation in Dextran 80. Partial replacement of sodium with divalent cations caused an increase of RBC aggregation and a decrease of disaggregation (Fig. 7). On an equimolar basis, there was no detectable dif-
ference among the effects of Mg++, Ca++, and Ba++ over the concentration range studied. The influence of divalent cations on RBC aggregation was also demonstrated by the increases in erythrocyte sedimentation rate (Fig. 8) and low-shear viscosity of normal RBC suspensions (Fig. 9). Neuraminidase-

![Diagram](image1)

**Figure 7.** Effects of various divalent cations on aggregation of normal RBC's in saline plus dextrans. The figure gives the relation between microscopic aggregation index (MAI) and dextran concentration. (A) and (B) show results with Dx 40 and 80, respectively.

![Diagram](image2)

**Figure 8.** Effects of CaCl₂ on sedimentation curves of normal RBC's suspended in saline plus 4 g/100 ml Dextran 80. Hematocrit = 45%.
treated RBC's showed stronger aggregation in Dextrans 40 and 80 than normal RBC's in the absence of divalent cations, and the degree of aggregation of the charge-depleted RBC's was not affected by the addition of divalent cations (Fig. 10). Results on sedimentation rate and viscosity measurement also indicated that divalent cations had no detectable effect on dextran-induced aggregation of neuraminidase-treated RBC's.

**Figure 9.** Effects of various divalent cations on suspension viscosities of normal RBC's in saline plus 4 g/100 ml Dextran 40 (A) and Dextran 80 (B) at 45% hematocrit.

**DISCUSSION**

The surface potential of normal RBC's results primarily from the ionogenic carboxyl groups of sialic acids (8, 9). Since the pK value of sialic acids is 2.6 (10), all sialic acids at cell surface should be almost completely ionized at pH = 7.4 and contribute to the negative charge of RBC surface. Calculations of the data obtained by Howe et al. (11) on isolated protein components of RBC membranes indicate that each sialoprotein molecule contains approximately 50 molecules of sialic acids. As sialoproteins align at the surface of RBC's, it is conceivable that the sialic acid molecules are located at different
depths of the membrane and that molecules situated at the surface of shear may contribute more strongly to the zeta potential than those positioned more internally.

1. Ionic Strength of the Suspending Medium

The contribution of the ionized sialic acids to the zeta potential is reduced by the presence of cations in the suspending medium (12, 13). This influence of cations is demonstrated by the decrease in the zeta potential of RBC's when the salt concentration and/or the cationic valency are increased in the suspending medium (Figs. 1 B and 6 B).

Theoretical calculation of the repulsive force between two similarly charged surfaces (1, 2) has shown that:

$$\Pi = [64 \varepsilon RT \tanh^2 (\zeta \psi_s/4kT)] e^{-d},$$  \hspace{1cm} (2)

where \(\Pi\) is the repulsive force per unit area, \(\varepsilon\) and \(\zeta\) are, respectively, the ionic concentration and valency, \(R\) is the gas constant, \(T\) is the absolute temperature, \(\psi_s\) is the surface potential, \(k\) is the Boltzmann constant, and \(d\) is...
the distance of separation, and \( \kappa \) is the slope of the potential drop in the double layer which can be calculated as:

\[
\kappa = \left( \frac{8\pi e^2 N c^2}{1000 D \kappa T} \right)^{1/2},
\]

where \( e \) is the electronic charge, and \( N \) is Avogadro's number. Hence a lowering of ionic strength causes not only an increase in surface potential (Fig. 1 B) but also a decrease in \( \kappa \) (Eq. 3). Both of these changes would enhance the electrostatic repulsive force between adjacent RBC's (Eq. 2) and result in a decrease in cell aggregation (Fig. 2).

For a fluid medium containing uni-univalent electrolyte (e.g. NaCl), the relation between electrostatic repulsive force and intercellular distance is illustrated in Fig. 11. The repulsive force between normal RBC's at 20 nm separation may increase from \( 3.01 \times 10^{-2} \) to \( 1.00 \times 10^3 \) dynes/cm² as the ionic strength is reduced from 150 to 50 mM. This marked increase in repulsive force (\( \sim 3 \times 10^4 \)-fold) can explain the total lack of RBC aggregation in Dextran 80 when the ionic strength is reduced to 50 mM (Fig. 2). The importance of the electrostatic repulsive force in influencing aggregation of
normal RBC's is further supported by the finding that variations in ionic strength between 50 and 150 mM have no effect on the aggregation of neuraminidase-treated RBC's in Dextran 80 (Fig. 5).

2. Ionic Species of the Suspending Medium

The present measurements on electrophoretic mobilities indicate that the screening of surface charge by divalent cations caused a greater reduction of zeta potential than the monovalent cations (Fig. 6). Theoretical considerations have shown that, for a given valency, the larger ions are more polarizable and effective in charge screening. Therefore one might predict, within each valency group, an order of cations in their effectiveness of screening (2): \( \text{Ba}^{2+} > \text{Ca}^{2+} > \text{Mg}^{2+} \) and \( \text{Na}^+ > \text{K}^+ \). Such specificities of cations of a given valency, however, are not demonstrable over the concentration range studied.\(^1\)

Since RBC's are negatively charged with no detectable cationic groups (8), charge screening by cations should predominate over that by anions. Heard and Seaman (4) have tested a series of sodium salts of different monovalent anions (NaF, NaCl, NaI, and NaSCN) and found no difference in RBC mobilities in these media. The present experiments on MgSO\(_4\) and MgCl\(_2\) (Fig. 6) indicate that even the variation in valency of the anion had no effect on the electrophoretic behavior of RBC's. Therefore, it may be concluded that the anions in the suspending medium do not play a significant role in determining the zeta potential of normal RBC's.

According to Eq. 2, the decrease in surface potential of normal RBC's and the increase in \( \kappa \) (or the shrinkage of double layer) caused by divalent cations should reduce the electrostatic repulsive force and favor RBC aggregation. Indeed, aggregation of normal RBC's in Dextran 40 and 80 is enhanced by the addition of divalent cations (Fig. 7), and the effect is similar for Ba\(^{2+}\), Ca\(^{2+}\), and Mg\(^{2+}\). The dextran-induced aggregation of neuraminidase-treated RBC's, which have already lost most of their surface charge, is not affected by the addition of divalent cations (Fig. 10). These results, therefore, again demonstrate the role of electrostatic repulsive force between cell surfaces in affecting the aggregation of normal RBC's.

3. Mechanism of RBC Disaggregation by High Concentrations of Dextrans

In the accompanying paper (1), it has been shown that dextrans cause an increase of zeta potential of normal RBC's in saline and the effect varies directly with dextran concentration and molecular size. The present experiments indicate that an increase in zeta potential is also obtained by decreasing the ionic strength (Fig. 1). High concentrations of Dextran 80 cause RBC disaggregation, and the dextran concentration required to achieve this effect is decreased at low ionic strength (Fig. 2), suggesting a synergistic...
action of these two disaggregating influences. By virtue of its molal volume, high concentrations of dextran cause a significant reduction in the volume available to water and electrolytes in the suspending medium (14). This volume exclusion effect of the neutral macromolecule may cause a reduction in effective ionic strength and an increase in electrostatic repulsive force between cell surfaces, thus leading to RBC disaggregation.

A consideration of the model of RBC aggregation by macromolecular bridging (3) and the results of the present study indicate that the relation between dextran concentration and RBC aggregation is determined by two opposing factors: as the dextran concentration increases, the increase in dextran bridges available would enhance RBC aggregation, but the concurrent increase in electrostatic repulsion due to the reduction in effective ionic strength would favor RBC disaggregation. The balance between these two factors results in the biphasic influence of dextran on RBC aggregation, i.e., RBC aggregation first rises to a maximum and then declines after the optimum dextran concentration has been exceeded (1). Increasing the ionic strength or the divalent cation concentration reduces the electrostatic repulsive force between cell surfaces and thus alters the balance between the two opposing factors affecting RBC aggregation by dextrans.

This investigation is part of a Dissertation submitted by K.-M. Jan in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Faculty of Pure Science, Columbia University.

The authors wish to acknowledge the valuable technical assistance of Ignacio Alvarez de la Campa, Dagmara Igals, Orlando M. Leyva, and Juan Rodriguez, and the excellent secretarial help of Miss Ethel M. Goodrich.

This investigation was supported by Research Grant HL-06139 from the National Heart and Lung Institute, the U. S. Army Medical Research and Development Command Contract DADA-17-72-C-2115, and by generous gifts from The Scaife Family Charitable Trusts of Pittsburgh, Pa., and Mrs. George W. Perkins.

Dr. Kung-ming Jan is a Postdoctoral Fellow of the New York Heart Association.

Received for publication 10 July 1972.

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