ELECTROPHORETIC STUDIES ON HUMAN RED BLOOD CELLS

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These experiments were made to obtain more information about the surface of the red blood cell. Since “surface” means different things to different people, we shall define our meaning now. Strictly speaking, the surface studied by electrophoresis is the surface of shear between the cell (moving in the electric field) and the surrounding medium, for it is the potential at this surface which is the electrokinetic potential. But since this potential has its origin in the charged groups possessed by the cell membrane, we shall broadly use the term surface (unless we say otherwise) to mean that part of the membrane possessing these charged groups. That part of the membrane may be limited to the outermost constituent molecules of the membrane (i.e., to those molecules just inside the surface of shear), but we have no direct evidence of this. Also we have no data on the variations of charge density in different portions of the surface.

The electrophoresis studies in this paper are divided into three parts. First, we have determined the mobility of human red cells as a function of the ionic strength at approximately constant pH. Secondly, we have determined the mobility as a function of pH at constant ionic strength for intact red cells, for the lipid of the red cell stroma, and for the protein of the stroma. Finally, we have determined the mobility of cells and ghosts under experimental conditions which cause changes in the mobility.

Methods and Preparations

1. Method of Electrophoresis.—The mobility measurements were carried out in an Abramson horizontal microelectrophoresis cell, using the technique of Abramson (1929, 1934) and of Moyer (1936). The cell was modified in two respects. The horizontal observation chamber dipped slightly below the level of the stopcocks and glass supporting rods, so that it rested on the microscope stage just above the condenser. This made it possible to use the cell with dark-field illumination (paraboloid) as well as with direct light. With direct light a Zeiss 28x ocular and 40x water-immersion objective were used, while with dark-field illumination we used the same ocular and a 20x high-dry objective. In the mobility measurements direct illumination was used except when specifically stated.

The other modification of the cell was the shortening of the vertical outlet tube over
one of the three-way stopcocks so that its top was a few centimeters below the top of the funnel-shaped inlet tube over the other three-way stopcock. We filled the cell rapidly while it was on the microscope stage in position for measurements. The cell, previously filled with the same solution as used for suspending the erythrocytes, was placed on the stage with the stopcocks adjusted to connect the inlet and outlet tubes by way of the observation chamber, and then the suspension of erythrocytes was poured into the inlet tube. This caused the solution originally in the cell to overflow out of the shortened outlet tube, while the suspension of erythrocytes in turn filled the cell. By pouring in a suspension immediately after making it, and by having electrical and optical adjustments for measurements approximately made before, we could make mobility measurements on the erythrocytes within 30 seconds of suspending them, which proved useful as we were often confronted with changes in mobility with time.

The mobility measurements were made at the "stationary levels." The specific resistance and the pH of each suspension were determined. The mobility measurements were made at room temperature, but all the mobilities were corrected to 25°C. Before each series of measurements the electrophoresis cell was cleaned with a concentrated Na3PO4 solution, and then, to ensure a uniform electroosmotic flow along the inside walls, a solution of serum was allowed to stand in the cell long enough for the walls to take on a coating of adsorbed protein.

2. Solutions.—What may be considered the standard reference solution for these experiments was an approximately isotonic mixture of nine parts of 1 per cent NaCl, 0.2 parts of M/15 KH2PO4, and 0.8 parts of M/15 Na2HPO4. This solution has an ionic strength of 0.172, a pH of 7.32 ± 0.2, and a specific resistance of 59.5 ± 0.3 ohms at 25°C. This will be called the "standard saline-phosphate solution."

For determining the effect of ionic strength on mobility, a series of mixtures of this standard solution and a 5.4 per cent glucose solution was made, containing per 100 parts respectively 50, 25, 10, 5, 4, and 2.5 parts of the standard solution. The pH values of the suspensions made with these mixtures were all over 7.0, except in the case of the last, where the pH was 6.85.

For the determination of the effect of pH on mobility at constant ionic strength, a series of mixtures of NaCl solution and various buffer solutions was made with the same ionic strength (0.172) as the standard saline-phosphate solution. The buffer solutions generally contributed about 0.1 of the total volume of the mixtures. The buffer systems were: M/10 NaOH—glycine—NaCl; M/15 Na2HPO4—KH2PO4; M/10 NaAC—HAc; M/10 HCl—glycine; and 0.13 M HCl.1

3. Preparation of Cells and Cell Products.—We obtained our cells from human blood drawn within a few hours of the electrophoresis measurements. It was mixed with about 100 times its volume of 1 per cent saline. The cells were centrifuged down, resuspended with 1 per cent NaCl in a conical centrifuge tube, and again centrifuged. The supernatant fluid was pipetted off. In making a suspension, enough cells were transferred on a stirring rod from the bottom of the centrifuge tube to the suspending solution so as to make a concentration of about 1 in 2000.

The lipid of the stroma was obtained by extracting intact cells at room temperature with a 3 to 1 mixture of ethyl alcohol (95 per cent) to diethyl ether, as was successfully

1 All systems to be found in Clark's The determination of hydrogen ions, Baltimore, The Williams & Wilkins Co., 3rd edition, 1928.
used by Boyd (1936) and by Dziemian (1939). Intact cells were used, for it has been shown (Beumer and Bürger, 1912; Erickson et al., 1938) that practically all the lipid of the red cell is contained in the stroma. The cells were obtained from freshly drawn, defibrinated human blood, and after being washed and packed, a few cubic centimeters were shaken with 30 volumes of the alcohol-ether solution for an hour. The mixture was then centrifuged, and the clear supernatant solution decanted into a suction flask. From the flask the solvent was evaporated under reduced pressure between 40 and 45°C., so that the extracted lipid was left as a thin skin on the bottom of the flask. By shaking the desired suspending solution in the flask and using a stirring rod to help to dislodge the lipid from the bottom, an emulsion of the lipid suitable for electrophoresis measurements can easily be made.

In obtaining the protein of the red cell stroma, washed ghosts were first prepared. Following the method of Parpart (1940), about 10 cc. of defibrinated and freshly drawn human blood were hemolyzed with 2 volumes of distilled water, and then about 20 volumes of CO₂-saturated water at about 0°C. were added to the mixture. The flocculated ghosts were centrifuged down, and washed six times at room temperature with a 0.05 per cent NaCl solution. The supernatant fluid from the last washing, completed within an hour of the original hemolysis, appeared to be free of hemoglobin, although the ghosts, being of a pale pink color, contained a little of it. In order to extract the lipid from the protein of the ghosts, the packed, washed ghosts were shaken with 30 volumes of a 3 to 1 alcohol-ether mixture at room temperature for about 1 hour. The residue from the extraction (the protein of the stroma) was then separated from the extracting solution by centrifugation, washed a few times with an alcohol-ether mixture, and dried in a current of air. For the purpose of electrophoresis measurements, small portions were shaken vigorously with the desired suspending solution, so that we obtained small fragments suitable for the making of measurements.

**RESULTS**

1. **Mobility as a Function of Ionic Strength**

In Table I are shown the mobilities of human red cells in mixtures of various ionic strengths and approximately constant pH (7.3–7.0 for all

<table>
<thead>
<tr>
<th>Ionic Strength</th>
<th>1/e X 10⁸</th>
<th>V</th>
<th>V, corrected for viscosity</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.172</td>
<td>7.37</td>
<td>-1.03</td>
<td>-1.04</td>
</tr>
<tr>
<td>0.086</td>
<td>10.40</td>
<td>-1.24</td>
<td>-1.35</td>
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<td>0.043</td>
<td>14.71</td>
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<td>-1.92</td>
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<td>0.017</td>
<td>23.12</td>
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<td>0.0086</td>
<td>32.95</td>
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<td>0.0069</td>
<td>36.80</td>
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</tr>
<tr>
<td>0.0043</td>
<td>46.50</td>
<td>-3.16</td>
<td>-3.63</td>
</tr>
</tbody>
</table>
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Fig. 1. Corrected mobility of human red cells as a function of $1/\kappa$.

Mobility as a function of pH for human red cells (crosses), for the lipid extract of the cells (open circles), and for the stroma protein of the cells (closed circles). This is at 25°C. and at ionic strength of 0.172.
except the mixture with an ionic strength of 0.0043 and pH of 6.85). In the second column of the table we have given the value of 1/κ for each mixture, where κ is the Debye function of ionic strength. (At 25°C., κ is $0.328 \times 10^4$ of the square root of the ionic strength.) In the fourth column of Table I we have given the mobilities corrected for viscosity, obtained by multiplying the observed mobilities of column 3 by the relative viscosities of the respective solutions used. In Fig. 1 are plotted the data of the second and fourth columns in Table I.

2. Mobility As a Function of pH

In determining the mobilities of human red cells at various pH levels and constant ionic strength, a phenomenon first emphasized by Abramson (1930) has to be considered. This phenomenon is the variation of the mobility with time in suspensions of low pH (generally under pH 4). However, after numerous observations, using the technique (see Methods) which allows for the making of the first mobility measurements within 30 seconds, we found that the mobility remains substantially constant for about 3 minutes from the time of the first observation (except when lysis by acid occurred within this short time, in which case the mobility remained constant until the lysis: the mobility would then begin to vary in a manner which will be discussed in the next section). Therefore, in obtaining mobilities of red cells at any pH below pH 4, we made observations on a few suspensions at the desired pH, and then used only the mobility measurements made during the periods before the onset of the variations with time so as to obtain an average mobility for the particular pH.

Table II shows the mobilities of red cells at various pH levels at an ionic
strength of 0.172. Dark-field illumination was used in determining the mobilities at the two lowest pH levels in order that the mobilities of the ghosts formed by acid hemolysis could be observed also.

In Fig. 2 are plotted the data given in Table II, and also the pH-mobility points obtained for the lipid extract of the red cell stroma and for the lipid extracted protein residue of the red cell stroma. The mobility measurements on the lipid and protein (made with dark-field illumination) were not so satisfactory as those on the intact red cells. The coefficient of variation of a series of individual measurements (of time) on a single suspension in the case of these cell constituents was generally about ± 10 per cent, as compared with a coefficient of variation of about ± 5 per cent for a similar series of measurements on intact cells. In fact, the point approximately at the isoelectric point on the protein curve is the average of measurements not only on particles with no mobility, but also on particles with very small positive mobilities and very small negative mobilities, whereas the point at the isoelectric point on the curve for intact cells is at the pH where all the cells were apparently stationary in the electric field.

3. Variations in Mobility under Certain Conditions

(a) The variation of mobility of red cells with time was found at all pH levels listed in Table II below pH 4.7. At pH 4.7, or at any higher pH levels, no variation was found during observations on a single suspension (taking usually about 15 minutes). At pH 3.8, where the variation was first noticed, the negative mobility of the cells began to decrease about 3 minutes after the mixing of the cell suspension, and in 10 minutes had fallen to half of the original value. At that pH, as well as at pH 3.5 and pH 2.9, the decrease in mobility began to occur before hemolysis occurred. At pH 2.2, however, hemolysis occurred about 3 minutes after the mixing, and the first change in the mobility began simultaneously with the hemolysis. This change in mobility was not a comparatively gradual decrease as was found at higher pH levels. The ghosts formed by the hemolysis from cells moving with a negative mobility began moving almost at once with a positive mobility. Likewise at pH 1.7, where the intact cells were stationary in the electric field, the ghosts formed by hemolysis (which was complete at this low pH within a minute) began to move at once with a positive mobility. The average positive mobility of the ghosts formed at this

*This coefficient of variation for measurements on intact cells is to be attributed more to errors in timing than to variations in mobilities. The individual mobility measurements were generally only about 7 seconds in length, and the stop-watch used recorded only 0.2 second intervals.*
lowest pH was 1.0 μ/sec./volt/cm., and did not appear to change during several minutes following hemolysis. (Similar mobility reversals on hemolysis in acid solutions had formerly been observed by Abramson (1930) with sheep cells.)

(b) A decrease of the negative mobility of intact red cells with time also occurs when the suspending solution is a mixture of 97.5 parts of 5.4 per cent glucose and 2.5 parts of the standard saline-phosphate solution. This decrease was decidedly slower than any caused by low pH. It involved a fall from −3.16 to −2.93 μ/sec./volt/cm. in 1 hour, and to −1.86 μ/sec./volt/cm. in 2 hours.

(c) Finally, one other change of mobility should be mentioned. This change was a decrease in the mobility of ghosts prepared by Parpart's method (1940) (see Methods). It has been previously shown by us (Abramson, Furchgott, and Ponder, 1939) that unhemolyzed rabbit red cells and ghosts made by various forms of lysis without subsequent CO₂-flocculation have the same mobility in a solution of glucose and phosphate buffer. Here we found that the ghosts of human red cells made by hypotonic lysis without subsequent CO₂-flocculation have the same mobility (−1.04 μ/sec./volt/cm.) as unhemolyzed human red cells in the standard saline-

In the case of the measurements at low ionic strengths (10 parts or less of standard saline-phosphate solution in 100 parts of the suspending mixture), it was found that small traces of CuSO₄ (of the order of 0.001 per cent in the case of a suspension containing 5 parts of the standard saline-phosphate solution per 100 parts) were capable of markedly and rapidly decreasing the red cell mobility, sometimes even to the extent of reversing the sign of the mobility. The pH decreases caused by such traces of copper salt were only about 0.1 of a pH unit and therefore of no consequence. The mobility decreases of this sort were first encountered when, because of inadequate washing of the electrophoresis cell before filling it with an erythrocyte suspension, traces of CuSO₄ from the electrode plugs contaminated the suspension. Somewhat similar mobility decreases in the presence of Cu⁺⁺ and certain other metal ions had been previously observed by Northrop and Freund (1923) and by Oliver and Barnard (1924).

Traces of CuSO₄, however, did not change the mobilities of the cells in the mixtures of standard saline-phosphate solution and 5.4 per cent glucose solution of higher ionic strengths (e.g., in a 1:1 mixture of these solutions). This was also the case when a solution of unbuffered 1 per cent saline was used as the suspending medium. Apparently the surfaces of the cells in the solutions of high glucose and low salt content were somehow changed from what they were in solutions of high salt content, so that they were capable of adsorbing cupric ions, thus changing their electrokinetic potential. Also there was no mobility change with traces of CuSO₄ in a mixture of 90 parts of glucose solution and 10 parts of M/15 phosphate buffer (pH 7.38). In this case the cupric ions, despite the relatively low total salt content, probably are prevented from being adsorbed on cells in detectable quantities because of the formation of poorly ionized complexes between them and the relatively abundant phosphate and acid phosphate ions.
phosphate mixture, whereas ghosts subjected to the CO₂-flocculation and 
washing of Parpart's method have a mobility of only $-0.85 \mu$/sec./volt/cm. 
in the same mixture. Ghosts prepared by Parpart's method also behave 
differently from ghosts hemolyzed by hypotonicity which have been subjected 
to less drastic treatment in that they do not disintegrate into stromatolytic 
forms on treatment with solutions of lyotropic salts such as lithium per-
chlorate (Furchgott, 1940).

**DISCUSSION**

From the results of the mobility measurements at various ionic strengths 
we obtain information about the contour of the red cell surface. Let us 
consider the curve in Fig. 1 in the light of Gorin's recent equations (Abram-
son, Gorin, and Moyer, 1939). Gorin's general equation (his equation 2') 
when applied to the limiting case of particles of very large radius of curva-
ture, gives us

$$V = \sigma(1/\kappa + \tau_i)$$  \hspace{1cm} (1)

where $V$ is the mobility in $\mu$/sec./volt/cm. corrected for the viscosity of the 
medium, $\sigma$ is the charge density of the surface of the particle, $\kappa$ is the Debye 
function of the ionic strength, and $\tau_i$ is the mean of the radii of the ions in 
the diffuse double layer. This equation predicts that for particles of very 
large radius of curvature $V$ is a linear function of $1/\kappa$ if the charge density 
remains constant.

However, for some particles which microscopically appear to have large 
enough radii to satisfy equation (1), measurements over ionic strength 
ranges in which the charge density varies inappreciably give non-linear 
$V$-$1/\kappa$ curves. From evidence obtained largely with microscopic particles 
coated with adsorbed protein, it appears that these non-linear curves are 
the result of "bumpy" surfaces, with the effective radius of curvature 
possibly being the radius of curvature of the individual bumps (Abramson, 
Gorin, and Moyer, 1939). Conversely it appears that for particles of large 
"gross" radius of curvature and constant charge density, deviation from 
linearity of the $V$-$1/\kappa$ curve may indicate a bumpy surface.

Looking back to Fig. 1 now, we see that below $1/\kappa$ of about $20 \times 10^{-3}$ 
(equivalent to an ionic strength of about 0.02), $V$ is a linear function of $1/\kappa$. 
Assuming that the charge density is almost constant over this range, our 
curve shows that the red cell surface behaves at ionic strengths above 0.02 
as a smooth surface with a very large radius of curvature. Further evidence 
for the applicability of equation (1) to the present data is the value of $\tau_i$ 
obtained by dividing the intercept of the curve by the linear slope. The
value is 1.8 Å, which is of the right order of magnitude for the mean of the radii of the ions (mostly Na⁺ and Cl⁻) in the diffuse double layer (Gorin, 1939).

At values of 1/κ above about $20 \times 10^{-8}$ (μ values below about 0.02), the curve in Fig. 1 is no longer linear. This, in our opinion, indicates that changes in the surface of the cell occur in solutions of μ < 0.02. A change from a smooth to a bumpy surface would decrease the slope of the curve, but it is more likely that the falling of the slope in Fig. 1 is a result of a decrease in charge density with the decrease of ionic strength. Such decreases of charge density in solutions of low ionic strength are a well-known phenomenon with various kinds of surfaces (Abramson and Müller, 1933). Other observations lead us to believe that the falling off of the slope of the curve in solutions of high glucose and low salt concentration may actually be connected with injury to the cell surface. By this we mean a change in the kind of molecules or in the arrangement of molecules in the surface.

Turning now to the pH-mobility curves in Fig. 2, the interesting point is the low isoelectric point of the red cell surface. This isoelectric point and also other points on the same curve below pH 4.0 were obtained before the onset of changes in mobility encountered at low pH levels. These changes in mobility with time, which may be due to adsorption of proteins (possibly hemoglobin where hemolysis is occurring) on the cell surface at low pH levels, have led to the reporting of wrong isoelectric points for red cells. Abramson (1930) has previously discussed this matter in some detail.

White and Monaghan (1936) have reported isoelectric points for ghosts (made by a somewhat drastic method of preparation) and lipid-extracted ghosts of cow, dog, and rabbit cells, but they did not make their measurements at corrected (for the “lens effect” of the cylindrical cell which they used) stationary levels. The isoelectric point which we have obtained for the lipid-extracted ghosts of human red cells (i.e., for the stroma protein) is (Fig. 2) at about pH 4.7. This is not an unusual isoelectric point for a protein, and is not in disagreement with the amino acid analyses which have been made on stroma protein (Jorpes, 1932; Beach et al., 1939).

Not only the isoelectric points, but also the entire pH-mobility curves for intact human red cells and for the stroma protein from them are decidedly different. Obviously the red cell surface is not a surface of stroma protein. The curve for the extracted lipid of the red cells is somewhat closer to that for the intact cells, but here again there are definite differences. The isoelectric point (by interpolation) of about pH 2.6 for the lipid is almost one pH unit higher than the isoelectric point of the cells, and the
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The electrophoretic mobility of unhemolysed human red cells has been determined as a function of ionic strength at approximately constant pH in isotonic mixtures of glucose solution and saline-phosphate buffer solution.

1. Above an ionic strength of about 0.02 the cells behave as particles with a smooth surface of large radius of curvature. Below an ionic strength of about 0.02, changes of the surface occur, probably involving a decrease of charge density and perhaps connected with injury of the surface.

2. The mobility as a function of pH at an ionic strength of 0.172 has been determined for human red cells, for the lipid extract of the cells, and for the stroma protein of the cells. The isoelectric points of cells, lipid, and protein have been found to be about 1.7, 2.6, and 4.7 respectively.

3. The pH-mobility data lead to the conclusion that a red cell surface is composed largely of lipid and dominated by strong acid groups, possibly the phosphoric acid groups of cephalin molecules.

SUMMARY

To Dr. M. H. Gorin, now of the Magnolia Petroleum Co., Dallas, Texas, and to Dr. H. A. Abramson of the College of Physicians and Surgeons, Columbia University, we extend our thanks for their interest and suggestions relative to the work in this paper.

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