Direct Measurement Of Potential Difference Across The Human Red Cell Membrane

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DIRECT MEASUREMENT OF POTENTIAL DIFFERENCE
ACROSS THE HUMAN RED CELL MEMBRANE

by

Department of Biophysics

Submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy

Faculty of Graduate Studies
The University of Western Ontario
London, Canada
February, 1971

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II ODE TO A RED CELL

Erythrocyte, O Erythrocyte,
You Biophysicists' delight!
Your biconcave shape, your sphericity,
Have aroused Dr. Burton's curiosity.
Are you under pressure, do you communicate?
Does mini-cylinder diameter determine your fate?
Oh, how I wish the Stokes and Nernst equations
Could solve the problems with accurate predictions,
The way you fall under gravity,
And the mystery of your electricity.

A.W.L.J.
This work was supported by the Medical Research Council of Canada. The author wishes to express his appreciation to this organization.
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ABSTRACT

The electrical potential difference across the human red blood cell membrane has been measured using ultra-fine glass microelectrodes filled with 3M-KCl solution. A rapid-advance mechanism was designed and used to drive the microelectrodes into the cells to allow instantaneous recording of the transmembrane potential.

The membrane potentials in red cells ranged from -1mV to -13mV, the cell interior being negative with respect to the modified Ringer solution in the extracellular phase. The mean value of -5.6mV agrees with the theoretical value calculated by the Nernst equation from the transmembrane chloride ratio, if 20 per cent of the intracellular water is unavailable as solvent to the chloride ions. This appears to support the hypothesis that 20 per cent of the red cell water is apparently non-osmotic.

The change in liquid-junction potential as the microelectrode is moved from Ringer solution to the cell cytoplasm has been estimated. It is most likely that our measurements were low, but by a maximum of 0.5mV.
The age dependence of the membrane potential has been studied. Cell populations of different mean cell ages were separated according to density by centrifugation. The study of separated and remixed cell populations showed that the separation procedure does not change the membrane potential. With the exception of the very young cells, the membrane potential decreases with cell age. The reason for a lower mean value obtained for the young cells has been discussed in terms of possible reticulocyte contamination.

Measurements on crenated cells gave small positive potentials. The cells crenated upon contact with glass surfaces. The reversal of the membrane potential has been discussed in terms of pH change and water loss.

Studies of aggregates of red cells in plasma also gave positive deflections. It is believed that the cell interior is still negative with respect to the immediate environment, but the effect of fixed charges on the surface of the aggregates and in the intercellular space is responsible for the apparent reversal in sign.

White cells were also studied. A mean value of -4.5mV obtained is in good agreement with results recently reported in the literature. Semipermeable microcapsules enclosing hemoglobin solutions have also been studied. The measured potential across the nylon membranes compares
well with the calculated Donnan potential. This further confirms the insignificance of any change in the liquid-junction potential as the microelectrode enters an environment where hemoglobin is present.
I. INTRODUCTION

Studies of membrane phenomena often involve the study of ionic fluxes through the membrane, the ionic concentrations, and the activity coefficients of the ions in both the intracellular and extracellular media. An expression relating these factors to the membrane potential has been derived (Ussing, 1949; Teorell, 1949), and is given for a passively distributed ionic species in the system by:

\[
\frac{\Phi_{\text{in}}^x}{\Phi_{\text{out}}^x} = \frac{[X]_o}{[X]_i} \cdot \frac{\gamma_o}{\gamma_i} \cdot \exp \left( \frac{ZF}{RT} \cdot E \right)
\]

In this equation, \(\Phi_{\text{in}}^x\) and \(\Phi_{\text{out}}^x\) are the influx and outflux of the passively distributed \(x\) ions through the membrane, \([X]_o\), \([X]_i\) are the extracellular and intracellular concentrations of the \(x\) ions, \(\gamma_o\) and \(\gamma_i\) are the activity coefficients of the \(x\) ions in the extracellular and intracellular media respectively. \(Z\) is the ionic valency, \(F\) the Faraday equal to 96,500 coulombs per mole,
R and T are respectively the gas constant and the absolute temperature, and E is the transmembrane potential, $E_1 - E_0$.

A calculation of the flux ratio requires knowledge of not only the concentration ratio and the activity coefficient ratio, but also the transmembrane potential. Therefore, the direct measurements of potential differences across membrane systems are important and necessary.

Guggenheim has stated that - "the electrical potential difference between two points in different media can never be measured and has not yet been defined in terms of physical realities: it is therefore a conception which has no physical significance." (Guggenheim, 1929). The full force of this emphatic statement is not applicable to most biological situations (Cole & Moore, 1960), and certainly not to the red cell, since the two media separated by the membrane are not different because the solvent in each case is water and the major ions Na$^+$, K$^+$ and Cl$^-$ are present on both sides. Under these conditions, the potential difference across the membrane is measurable.

The red cell is in a steady-state under normal conditions, and the resting membrane potential is constant. The influx $\phi_{\text{in}}^x$ equals the outflux $\phi_{\text{out}}^x$ for each ionic species at steady-state conditions. In studies of membrane phenomena in the red cell, it has for many years been
customary to assume that the membrane potential can be calculated from the chloride concentration ratio using the Nernst equation for the equilibrium potential:

\[ E = \frac{RT}{2F} \ln \frac{[Cl^-]_o}{[Cl^-]_i} \]

This assumption implies that the chloride ions are passively distributed across the membrane, and that the ratio of the activity coefficients for chloride is unity. Since the red cell membrane is highly permeable to Cl\(^-\) (Tosteson, 1959), it is justifiable to assume a passive distribution of the chloride ions across the cell membrane. The red cell contains a concentrated solution of hemoglobin which is negatively charged. Under these conditions, it is not clear that the activity coefficients inside and outside the cell will be equal. Thus, there is uncertainty in the calculated membrane potential, and direct measurement of the membrane potential is desirable.

There are limitations in direct measurements of membrane potentials from red cells. Because of the small size of the cell, there are technical problems involved in the actual penetration of the membrane by measuring electrodes. As a result, steady potentials are difficult to obtain. The liquid-junction potentials also present a problem. As an electrode moves from a Na\(^+\) environment in the extracellular medium to a K\(^+\) environment
in the intracellular compartment, there is a change in the liquid-junction potential recorded in addition to the membrane potential. Since the relative contributions of the two are not known, an unknown error exists in the measurements. Part of this thesis is devoted to the development of technique in the use of ultra-fine glass microelectrodes for direct measurements of potentials in red cells. The initial results have been reported (Jay, 1968; Jay & Burton, 1969). The results obtained are in agreement with those by another group of researchers (Lassen & Sten-Knudsen, 1968) who carried out a similar independent study simultaneously with our work. This thesis also considers the possible error due to liquid-junction potential change calculated from extreme conditions and shows that the maximum possible error is less than one millivolt. This systemic error probably has caused our measurements to be low by a fraction of a millivolt.

The ionic distributions of Na\(^+\) and K\(^+\) across the red cell membrane are not at equilibrium but in steady-states maintained by the energy-expending active transport mechanism. This is a function of the membrane by which Na\(^+\) is extruded from the cell interior against a concentration gradient in exchange for K\(^+\). Deterioration of the active transport mechanism as the cell ages
during its life span will cause changes in the Na\(^+\) and K\(^+\) gradients (Prankerd, 1958; Bernstein, 1959). As a result, the gradient of the passively distributed Cl\(^-\), and therefore the membrane potential, may also change.

This thesis also presents a direct investigation of the change in membrane potential as the cell ages during its in vivo life span.
II. THERMODYNAMIC BASIS OF BIOELECTRICITY

(1) INTRODUCTION

The bioelectric phenomena in living systems are associated with the electric charges carried by ionized molecules and ions. Electric potential differences within organisms, and between organisms and their environment, are due to unequal distribution of these charged particles. Although biological systems are often too complicated for complete understanding and analysis, the principles of thermodynamics have been able to explain many of the observed biological phenomena, such as the behavior of enzyme systems, transport of molecules against chemical and electrical gradients and the molecular basis of nerve conduction. There is virtually no field of biological science to which the concepts of "irreversible" thermodynamics cannot be applied.

This chapter is devoted to presenting some of the basic thermodynamic principles which are relevant to an understanding of "bioelectricity".
(2) DIFFUSION POTENTIAL

The removal of a partition between two compartments containing unequal concentrations of an electrolyte solution results in diffusion of the dissociated ions from high to low concentration. Because the dissociated cations and anions are different in size and mass, they have different diffusion velocities in the solution. The unequal diffusion velocities give rise to a separation of charged ions, and establish a potential difference between the two compartments. This is called a "diffusion potential" and is dependent on the difference in diffusion velocities, i.e. mobilities, of the two ions, on the concentration gradient, and on the absolute temperature.

Since the electric effects are caused by the diffusion, which in turn is caused by the difference in concentration, the electric work must be equal to the osmotic work. The diffusion potential can be calculated (Nernst, 1888, 1889).

The electric work $W_e$, in electrostatics, is given by

$$W_e = E \cdot ZF$$

where $W_e$ is the work that must be done solely against
the electric field, to transfer 1 mole of that ion from the lower to the higher potential while keeping the potentials constant at the original values. $E$ is the electric potential difference in volts, $Z$ is the valency of the ion and $F$ is the Faraday, 96,500 coulombs of charge.

The osmotic work $W_\Pi$, from thermodynamic principles is given by

$$W_\Pi = \left(\frac{u-v}{u+v}\right)RT \cdot \ln \frac{C_1}{C_2}$$

where $W_\Pi$ is the work required to transfer 1 mole of an ion solely against the concentration gradient, with the gradient held constant. $u$ and $v$ are the diffusion velocities of the cation and anion under unit force respectively. $R$ is the universal gas constant, 8.31 joules/mole/°K, $T$ is the absolute temperature and $C_1$, $C_2$ are the concentrations of the electrolytes in the two compartments.

If electric work is equal to osmotic work, then

$$W_e = W_\Pi$$

or,

$$E \cdot ZF = \frac{u-v}{u+v} \cdot RT \ln \frac{C_1}{C_2}$$
and the diffusion potential is given by

\[ E = \frac{u-v}{u+v} \cdot \frac{RT}{2F} \ln \frac{C_1}{C_2} \]

The diffusion potential is produced by an imbalance of ionic concentrations within the same solvent system, and is reduced to zero when equilibrium is reached, and \( C_1 = C_2 \).

(3) PHASE-BOUNDARY POTENTIALS

Electric potential differences can also develop at the boundary of two immiscible solutions when there are one or more ionic species common to both phases. Because the solubility coefficients of the solute are different in the two solvents, the system can attain equilibrium while ionic concentration gradients persist. Thus, a phase-boundary potential exists when the two phases are in equilibrium and the potential difference balances the diffusion tendency of the ions.

The phase-boundary potential (Beutner, 1920) can be calculated for each ionic species common to both phases,

\[ E = RT \ln \frac{C_1}{C_2} + K \]

where \( K \) is a constant specific for the particular solute,
C_1, C_2 are the molar concentrations of the ion.

In contrast to the diffusion potential, the phase-boundary potential is a steady potential which can be described as an equilibrium potential. Since at equilibrium no ions move, no current and therefore no work can be derived from the system.

Phase-boundary potentials are likely to exist whenever an aqueous medium borders on a nonaqueous one. In biological systems, such situations are frequent and must occur at most internal and external membranes of cells as well as at the borders of lipid inclusion bodies.

(4) THE GIBBS-DONNAN EQUILIBRIUM AND THE SEMIPERMEABLE MEMBRANE

Consider a cell with a semipermeable membrane containing negatively charged protein P^-, sodium Na^+ and chloride Cl^- ions. Suppose the membrane is permeable to the Na^+ and Cl^- ions but not the protein which is a much larger particle, and that the system is placed in a solution containing Na^+ and Cl^- ions. There will be movements of the permeating ions across the membrane, and the movements will continue until equilibrium is reached. The movements of the ions will be with the concentration
gradients due to diffusion. The situation however is not quite so simple. Oppositely charged ions attract each other and similarly charged ions repel each other. The tendency to establish equal concentrations of the ionic species must therefore compete with the tendency to establish electric neutrality.

Electric neutrality in both the intracellular and extracellular fluids will occur if

\[ [\text{Na}^+]_i = [\text{P}^-]_i + [\text{Cl}^-]_i \]

and

\[ [\text{Na}^+]_o = [\text{Cl}^-]_o \]

where the subscripts \( i \) and \( o \) represent inside and outside the cell respectively. It is obvious that

\[ [\text{Na}^+]_i > [\text{Cl}^-]_i \]

but

\[ [\text{Na}^+]_o = [\text{Cl}^-]_o \]

Once equilibrium is reached, there is no further net movement of ions across the semipermeable membrane. The amounts of work required to transport a small amount of \( \text{Na}^+ \) in one direction and an equal amount of \( \text{Cl}^- \) in the
opposite direction must be equal. The work required to transport \( \Delta n \) moles of \( Na^+ \) out of the cell and \( \Delta n \) moles of \( Cl^- \) into the cell are respectively given by

\[
\delta n \cdot RT \ln \frac{[Na^+]_i}{[Na^+]_o}
\]

and

\[
\delta n \cdot RT \ln \frac{[Cl^-]_o}{[Cl^-]_i}
\]

At equilibrium, these two must be equal, therefore

\[
\frac{[Na^+]_i}{[Na^+]_o} = \frac{[Cl^-]_o}{[Cl^-]_i}
\]

or,

\[
[Na^+]_i[Cl^-]_i = [Na^+]_o[Cl^-]_o
\]

This is known as the "Donnan rule" and assumes that all NaCl present in the system is completely dissociated.

Now, since \( [Na^+]_o = [Cl^-]_o \) and \( [Na^+]_i > [Cl^-]_i \)

from

\[
\frac{[Na^+]_i}{[Na^+]_o} = \frac{[Cl^-]_o}{[Cl^-]_i}
\]

it can be seen that

\[
[Na^+]_i > [Na^+]_o = [Cl^-]_o > [Cl^-]_i
\]
The total concentration of solutes inside the cell is given by

\[ [\text{Na}^+]_i + [\text{P}^-]_i + [\text{Cl}^-]_i = 2[\text{Na}^+]_i \]

and that in the extracellular medium is

\[ [\text{Na}^+]_o + [\text{Cl}^-]_o = 2[\text{Na}^+]_o \]

since \[ [\text{Na}^+]_i > [\text{Na}^+]_o \]

The total solute concentration at equilibrium conditions is greater inside the cell than outside, i.e.

\[ [\text{P}^-]_i + [\text{Na}^+]_i + [\text{Cl}^-]_i > [\text{Na}^+]_o + [\text{Cl}^-]_o \]

This is known as the "Donnan Equilibrium" (Donnan, 1911; Overbeek, 1956). The Donnan Equilibrium not only represents an osmotic imbalance but also a peculiar distribution of the diffusible charges. In spite of electric neutrality within each of the two fluid compartments, there are differences in concentrations of positive and negative charges in the two compartments. The differences in each of positive and negative charges give rise to electric potentials.
Since the differences are in opposite directions and the charges are opposite to one another, the resultant electric potential differences are parallel, i.e. have the same polarity.

(5) RESTING MEMBRANE POTENTIALS IN CELLS

When a voltmeter is connected with a suitable electrode inserted into a living cell and the other placed in the extracellular medium, a steady difference of potential up to approximately 100 millivolts is indicated. This steady potential is called the "resting potential" of the cell. For some type of cells, this potential difference can be altered artificially by changing the concentrations of certain ions, especially K⁺ and Cl⁻, in the external medium. From studies of frog muscles (Adrian, 1956), it has been calculated that the potential change follows approximately the logarithm of the ratios of the inside to outside concentrations of these ions. Furthermore, it has been shown that in nerve and muscle cells the ratio of the Cl⁻ concentrations approximates the reciprocal of that of the K⁺ (Hodgkin, 1951). The resting potential is not significantly affected by changes in the external Na⁺ concentration nor is it related to
the existing Na\(^+\) ratio. These findings led to the conclusion that the resting potential is determined largely by the K\(^+\) and Cl\(^-\) concentration differences (Boyle & Conway, 1941; Curtis & Cole, 1942; Hodgkin & Keynes, 1955). It had long been hypothesized that Na\(^+\) did not penetrate the cell membrane (Bernstein, 1912). The hypothesis was shown to be inaccurate when it was discovered that animal cells are actually permeable to Na\(^+\) although the permeability is lower than that to K\(^+\) and Cl\(^-\) (Hevesy, 1948), and that Na\(^+\) ions leave the cells at the same rate as they enter.

The new theoretical treatment of the resting potential given in the works of Hodgkin and Huxley (Hodgkin & Huxley, 1952 a-d) is widely accepted by present day physiologists. The situation is considered to be in a "steady-state" rather than in equilibrium. While the resting potentials in nerves and muscles are close to the equilibrium potentials of K\(^+\) and Cl\(^-\), they are not equal to those of Na\(^+\). The Na\(^+\) distribution across the cell membrane then is not an equilibrium distribution and the concentration energy difference for Na\(^+\) across the membrane is not balanced by the electric potential energy difference. The inward movement of Na\(^+\) is passive, with the concentration gradient and electric potential and
requires no energy, but the outward movement is against the concentration gradient and electric potential difference, and must be an active process which requires the expenditure of energy derived from metabolism of the cell. This "active transport" system balances the passive outward diffusion and maintains the steady-state Na⁺ distribution across the cell membrane.
III. THE HUMAN RED BLOOD CELL

(1) INTRODUCTION

The red cells make up 45 to 50 per cent of the total blood volume. There are approximately five million red cells in one cubic millimeter of blood. Leeuwenhoek in 1673 (see Wintrobe 1961, p. 85) was the first to discover the "red globules" and attributed the red color of blood to them. About one hundred years later Hewson recognized that the red globules are really flat discs rather than spherical globules. During the latter half of the nineteenth century, many studies of the red cell were made. Hoppe-Seyler in 1867 (see Wintrobe 1961, p. 85) demonstrated the oxygen carrying property of hemoglobin, and Neumann in 1868 (see Wintrobe 1961, p. 85) showed that the red cells are formed in the bone marrow.

The mature human red cell is devoid of any organelle. It contains mainly hemoglobin, water and electrolytes enclosed in a thin membrane. It is perhaps the most simple cell studied. Although the red cell is morphologically simple, its peculiar but amazingly uniform shape has baffled physiologists and biophysicists for half
a century. In recent years, studies on the geometry of the red cell have been extensive, (Ponder, 1948; Prankerd, 1961; Canham & Burton, 1968) but the explanation for its shape is still not completely resolved (Rand & Burton, 1964; Rand, 1964; Fung, 1966; Fung & Tong, 1968; Shrivastav & Burton, 1969; and Canham, 1970).

(2) GENERAL DESCRIPTION OF THE HUMAN RED BLOOD CELL

The mature human red cell has the shape of a biconcave disc. It consists of two components, a membrane, and a solution of hemoglobin and electrolytes. The concentration of hemoglobin is very high. Ponder (1948) was the first to study the shape of the red cell in detail and systematically. Results show that the human red cell has a mean diameter of 8.5μ, a mean greatest thickness at the rim of 2.4μ and a mean minimum thickness, which occurs at the dimple region, of 1.0μ. Calculations show the cell to have a mean area of 163μ² and a mean volume of 87μ³ (Fig. 1).

A more recent study by Canham and Burton (1968) confirms Ponder's value for the diameter, but the results show that the area is lower (138.1μ²) and the volume is higher (107.5μ³). The authors have shown that the three parameters, diameter, area and volume are not independent
FIGURE 1

Shape of the normal human red blood cell as viewed from the flat and from the edge (cross-section A-A\textsuperscript{1}).

Ponder (1948)

<table>
<thead>
<tr>
<th>Measurement</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Greatest thickness</td>
<td>2.4\textmu</td>
</tr>
<tr>
<td>Least thickness</td>
<td>1.0\textmu</td>
</tr>
</tbody>
</table>

Canham and Burton (1968)

<table>
<thead>
<tr>
<th>Measurement</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diameter</td>
<td>8.068\pm0.547(S.D)\textmu</td>
</tr>
<tr>
<td>Area</td>
<td>138.1\pm17.4(S.D)\mu^2</td>
</tr>
<tr>
<td>Volume</td>
<td>107.5\pm16.8(S.D)\mu^3</td>
</tr>
</tbody>
</table>
variables, and that cells with larger volumes are thinner, a necessary condition for survival in the circulation.

(3) METABOLISM OF THE RED CELL

While the principal task of the immature erythrocyte and its precursors is the biosynthesis of hemoglobin, that of the mature erythrocyte is the maintenance of the synthesized hemoglobin in a functional state. The transport of oxygen from the lungs to the tissues is made possible by the hemoglobin which binds the gas reversibly. For many years, it was believed that blood viscosity would be comparatively much higher (an undesirable condition), if the hemoglobin had been present in the circulation as a free solution instead of being enclosed in cells. This reason for the existence of the red cell was disproved when the converse was shown to be true (Trubowitz & Masek, 1968; Cokelet & Meiselman, 1968).

The incorporation of hemoglobin into red cells does, however, provide protection for the hemoglobin from extensive degradative activity and prevents its loss from the circulation through the capillary walls, especially in the kidney. The membranes of the glomerular capillaries in
the kidney have equivalent pores 75 to 100\text{"A} in diameter (Pappenheimer, 1955; see Pitts, 1963, p. 56), which are sufficiently large for the passage of hemoglobin molecules with an effective diameter of 65\text{"A} (see Pitts, 1963, p. 55).

The article by London (1960) gives a comprehensive review of the literature on the metabolism of the mammalian erythrocyte. The red cell is released from the bone marrow into the circulation (after loss of the nucleus) in the form of a reticulocyte. A major constituent of the reticulum is RNA (ribonucleic acid), making it possible for the reticulocyte to carry out hemoglobin synthesis (Kruh & Borsook, 1956). The reticulocyte has an active Embden-Meyerhof glycolytic cycle, an active hexosemonophosphate pathway and an intact Krebs tricarboxylic acid cycle.

In the mature erythrocyte, there is little or no RNA, and only slight remnants of the citric acid cycle remains. The synthesis of hemoglobin does not occur, and that of lipid is absent or insignificant in the mature erythrocyte (London & Schwarz, 1953; Reed, 1959; and Mark & Gellhorn, 1959).

Metabolism of the mature erythrocyte takes place in the membrane. The oxygen consumption of mammalian red cells on the basis of total weight, is very low compared to that of other cells and tissues, and is only 2 to 3 per cent
of that of reticulocytes, the non-nucleated immediate precursors of red cells (Ponder, 1948, p. 357). The oxygen consumption of the red cell membrane however, is comparable to that of other cell membranes. This implies that only the membrane of the matured erythrocyte is "alive".

The red cell contains no glycogen. Consequently, for its continued metabolism, it must have constant access to glucose. While the exact mechanism of how glucose gets into the red cell is unknown, investigators have concluded that it depends on an active transport mechanism rather than passive diffusion (LeFevre & Marshall, 1958; Widdas, 1954; Bowyer & Widdas, 1958). Mitochondria, the cytoplasmic organelles associated with energy metabolism in other types of cells are absent in the mature erythrocyte. The metabolic breakdown of glucose depends on the reactions of the Embden-Meyerhof glycolytic pathway whereby glucose is broken down anaerobically to lactic acid. An alternate pathway for the metabolism of glucose is via the "hexose monophosphate shunt" through which the sugar is oxidized with the formation of pentose and triose phosphates (Prankerd, 1955). The metabolism of glucose circulating in the blood serves two major functions: the maintenance of oxidation-reduction homeostasis through the formation of reduced pyridine nucleotides, and the generation of the
high energy compound ATP (adenosine triphosphate) required for active transport and the maintenance of the structural integrity of the erythrocyte.

(4) ELECTROLYTE COMPOSITION AND ACTIVE TRANSPORT

With the demonstration that it has a definite life span and metabolism, the erythrocyte is no longer thought of as an inert bag of hemoglobin which did not even merit the term "cell", and was therefore named a red blood "corpuscle".

The red cell contains large negatively charged molecules, mainly hemoglobin (but also some non-hemoglobin proteins, sugar phosphates, etc.) to which the cell membrane is not permeable. It also contains small ions, most importantly sodium, potassium and chloride, as well as water to which the cell membrane is permeable to different degrees.

The water contained in the red cell makes up 70 per cent of its volume or approximately 65 per cent of its weight (LeFevre, 1964; Gary-Bobo & Solomon, 1968). Most of the remainder is hemoglobin at a concentration of about 30 grams per 100 ml cells (Maizels, 1936; Altman & Dittman, 1964, p. 268). Biochemical studies have shown that potassium is the major electrolyte within the human red cell at a concentration of about 136 to 155 mEq/L cell H₂O, sodium
concentration is comparatively low at 17 to 25 mEq/L cell H₂O, while chloride is present at 73 to 89 mEq/L cell H₂O (Maizels, 1936; Snyder & Katzenelbogen, 1942; Bernstein, 1954; Keital et al., 1955). The ranges represent the variation of the results obtained by different investigators, and the values have been recalculated from the original data to give consistent units. Although it has been shown that all the water in the red cell serves as solvent for glucose (Miller, 1964) and other non-electrolytes (Gary-Bobo, 1967), it has long been known that only part of the water serves as solvent for electrolytes (Cristol & Benezech, 1946; Ørskov, 1947; Ponder, 1948). The osmotically inactive part of the cell water has been determined to be about 20 per cent of the total cell water (Savitz et al., 1964; Gary-Bobo & Solomon, 1968). This value agrees very well with the amount of "bound water" observed in studies of methemoglobin crystals (Perutz, 1946; Drabkin, 1950).

Thus, the effective concentrations of sodium, potassium and chloride in the intact cell may actually be higher than those reported by approximately 20 per cent. Nevertheless, since the plasma contains 150 to 160 mEq/L plasma H₂O sodium and only about 4 to 8 mEq/L plasma H₂O potassium (Snyder & Katzenelbogen, 1942), there are very large steady state concentration gradients of both sodium and potassium across the cell membrane.
The maintenance of large steady state concentration gradients of sodium and potassium across the cell membrane requires an active transport of ions against the gradients. Without such a mechanism, the gradients will decrease with time due to passive diffusion and eventually reach equilibrium values, i.e. Donnan Equilibrium. The movements of cations across the red cell membrane have generally been considered to be by three conceptually different membrane pathways (Hoffman, 1966). Cations can move "uphill" or against a concentration gradient by the active transport "pump"; "downhill" or with the gradient by passive diffusion "leak"; and by exchange diffusion. Net movements of ions can occur only through the pump and leak pathways, and it is the balance between these two that maintains the steady state gradients of each of the ions.

The work of Tosteson & Hoffman (1960) on high and low potassium sheep cells gives a very good demonstration of the balance between the active pump and passive leak mechanisms. Individual sheep in the same flock may have red cells which are either higher or lower in potassium than in sodium, while the plasma in both groups of sheep are high in sodium and low in potassium. This means that in the case of low K⁺ red cells, the transmembrane K⁺ and Na⁺ concentration gradients are several times lower than the usual values. As a result, the passive cation leakage
fluxes are comparatively lower. Tosteson and Hoffman have found that the active pumps in low K⁺ red cells are also comparatively lower, approximately by a factor of four. Thus, the two mechanisms of cation movements across the membrane are still in balance, and steady state concentrations are still maintained.

The operation of the active pump, which is a function of the membrane (Hoffman, 1962a), requires energy from ATP generated from glycolysis (Glynn, 1957a; Post et al., 1960; Hoffman, 1962b). The utilization of ATP is related to ATPase which has been found to be bound to the membrane (Post et al., 1960; Blostein, 1968). The action of ATPase, the enzyme which releases inorganic phosphate from ATP, has been shown to be stimulated by the presence of sodium and potassium and inhibited by cardiac glycosides (Glynn, 1957b; Hoffman, 1964).

(5) **LIFE SPAN OF RED CELLS IN VIVO**

The human red cell has a mean life span of 120 days in the circulation. In the normal subject, about 0.8 per cent of the cells are destroyed daily and newly formed cells replenish the population. A number of methods have been used in attempts to measure the life span of the red cell, (Berlin, et al., 1959). The most important are the
differential agglutination or Ashby technique and the isotopic method. The method of differential agglutination (Ashby, 1919, 1948) involves the transfusion of compatible but immunologically identifiable blood. Periodically, the donor red cells are enumerated following agglutination of the recipient's cells by appropriate antisera.

Isotopic methods include the use of radioactive tracers, e.g. $^{15}$N, $^{14}$C, $^{55}$Fe, $^{59}$Fe and $^{51}$Cr. Except for $^{51}$Cr, labelling of red cells is done in vivo. The use of glycine labelled with $^{15}$N or $^{14}$C has the important advantage that these elements are incorporated metabolically into red cells at a particular stage of their formation. However, the need for special equipment reduces the applicability of an otherwise satisfactory method. Radioactive iron procedures involve problems of reutilization and complex methods of analysis which seriously limit the utility of the method. The use of $^{51}$Cr is the most practical method of radioactive tagging of red cells (Donuhue et al., 1955; Mollison & Veall, 1955; Hugh-Jones & Mollison, 1956). Red cells can be labelled in vitro by $^{51}$Cr-labelled sodium chromate which becomes fixed in the red cells. They are given to a recipient and the rate of change of $^{51}$Cr concentration per ml of red blood cells is determined. There appears to be no reutilization of isotope released from the destruction of the cells.
Although the methods are different, the results are essentially the same. The mean life span of red cells was measured to be 117(110-135) days by the Ashby method, 113(108-120) days by the Cr$^{51}$-method and 118(109-127) days using N$^{15}$-labelled glycine.

(6) AGING OF RED CELLS IN VIVO

As the red cell ages during its life span of 120 days, it undergoes various changes physically, chemically as well as metabolically. The combined use of centrifugation and radioactive tracer techniques has shown that the density of the red cell increases with age (Borun et al., 1957; Danon and Marikovsky, 1964). Moreover, the relation between mean cell density and mean cell age is approximately linear (Leif & Vinograd, 1964; Piomelli et al., 1967). The separation of red cell populations into age groups, according to density, by centrifugation has become an important tool in studies of other characteristics of red cell aging. Centrifugation of red cells can be done in continuous or discontinuous density gradients, or it can be done in isotonic saline or Ringer solution. The continuous density gradient method fractionates red cells in a linear density gradient of bovine serum albumin (Leif & Vinograd, 1964). The column is prepared using a density gradient machine so
that the albumin solution has increasing density with depth of the tube. The cell suspension is layered onto the column and centrifuged. The discontinuous gradient method uses ester mixtures of known specific gravities which are not miscible with the aqueous extracellular medium. Upon centrifugation, the ester layer separates cells according to whether the density is higher or lower than its own (Danon & Marikovsky, 1964; Piomelli et al., 1967). Depending on whether the ester mixture is initially placed on top or bottom of the centrifuge tube, either the lighter or denser cells respectively will pass through the oil. Centrifugation of red cells in their own suspending medium, either isotonic saline or Ringer solution, requires washing the cells before separation in order to avoid rouleaux formation. The method has been used successfully in both large and small blood samples in studies of erythrocyte lipids (Westerman et al., 1963) and of acetylcholinesterase activity (Herz & Kaplan, 1965). Results of the study by Herz & Kaplan (1965) which compares the macro and micro techniques seem to indicate that the microtechnique gives better separation of cells. This method of separation has also proven to give better separation than the discontinuous gradient method in cell size and shape studies (Canham & Parkinson, unpublished data). The microtechnique method is used in this study and is described in detail in the Methods section.
In addition to the increased specific gravity, the aging process in the normal human erythrocyte is also accompanied by a variety of other biophysical and biochemical changes. In physical dimensions, older cells have slightly smaller diameters, surface areas and volumes, and are more spherical than young cells (Canham, 1969). They also have lower rates of anaerobic glycolysis and cation transport, and have lower potassium and higher sodium concentrations than younger cells (Bernstein, 1959; Prankerd, 1958). Lipid content decreases with cell age (Westerman et al., 1963), so do activities of catalase, cholinesterase (Allison & Burn, 1955), glucose 6-phosphatase (Marks et al., 1958) and erythrocyte glutamic oxalacetic transaminase (EGOT) (Bartos & Desforges, 1967). Older cells also have been shown to have less surface charge (Danon & Marikovsky, 1961; Marikovsky & Danon, 1969; Yaari, 1969) and are more susceptible to osmotic as well as immune lysis (Marks & Johnson, 1958; London, 1960).

Most of the changes with age described above, suggest a decrease in metabolic activity and efficiency of the active transport mechanism.
IV. THEORETICAL CONSIDERATION OF THE
RED CELL MEMBRANE POTENTIAL

(1) THE GOLDMAN CONSTANT FIELD EQUATION

Membrane potentials of animal origin were first
directly and accurately measured in the case of the giant
axons of squids Sepia and Loligo (Hodgkin & Huxley, 1939;
Curtis & Cole, 1940). The techniques were soon refined
sufficiently so that potentials can now be measured from a
variety of smaller cells including muscle and smaller
nerve cells. When the directly measured potentials are
compared to those calculated from the chemical contents of
the cells, it becomes evident that the measured values of
the potentials are usually slightly lower than those pre-
dicted by the Nernst equation for the equilibrium potentials
for $K^+$ and $Cl^-$ ions. This means that the situation is more
complicated than expected, and that a mathematical formu-
lation, more sophisticated than that provided by the Nernst
equation, is needed.

As pointed out in the discussion of the resting
potential, the measured potential although quite close to the equilibrium potential for $K^+$ and $Cl^-$, is far from that for $Na^+$. The distribution of $Na^+$ across the membrane therefore is not an equilibrium distribution but a steady-state resulting from a balance between active-transport and diffusion. The membrane is not completely impermeable to $Na^+$ and therefore $Na^+$ ions must influence the membrane potential.

The quantitative treatment of the situation is given by a modification of Goldman's constant field equation (Goldman, 1943) as developed by Hodgkin and Katz (Hodgkin & Katz, 1949):

$$E = -\frac{RT}{F} \ln \frac{P_K[K^+]_i + P_{Na}[Na^+]_i + P_{Cl}[Cl^-]_o}{P_K[K^+]_o + P_{Na}[Na^+]_o + P_{Cl}[Cl^-]_i}$$

In this equation, $E$ is the membrane potential (inside-outside), $P_K$, $P_{Na}$ and $P_{Cl}$ are the relative permeabilities of the $K^+$, $Na^+$ and $Cl^-$ ions respectively, and the other terms are as previously defined. This equation has been developed with four basic assumptions:

(1) the voltage gradient through the membrane is assumed to be constant, i.e. constant field;

(2) the ions move under the influence of diffusion and the electric field;
(3) the concentration of ions at the edges of the membrane are directly proportional to those in the aqueous solutions, and that

(4) the membrane is homogeneous.

This hypothesis considers all three major ions present in most biological systems, including the non-passively distributed Na\(^+\) ions, by taking into account their relative permeabilities. The equation has been shown to be satisfactory in the study of resting and action potentials in the giant squid axons (Hodgkin & Katz, 1949) when appropriate permeability ratios are used. The expression is applicable to other biological systems and provides an approximation for the membrane potentials in red cells when further simplifications are made.

(2) THE NERNST EQUATION AND THE CHLORIDE POTENTIAL

The distribution of the permeant ions across the red cell membrane does not constitute a Donnan equilibrium. This is due to the presence of the active-transport mechanism, the Na\(^+\) - K\(^+\) exchange pump. Because of the presence of the impermeant protein hemoglobin at a very high concentration, Donnan equilibrium predicts higher osmotic pressure inside than in the medium, and an internal hydrostatic pressure of about 6 x 10\(^3\) mm H\(_2\)O in excess of the external pressure
(Tosteson, 1967). However, it has been shown that there is no significant osmotic gradient across the membrane (Conway & McCormack, 1953; Appelboom et al., 1958) and the internal pressure has been measured to be only about 2 mm H₂O (Rand & Burton, 1964).

The deviation from Donnan equilibrium conditions is due to the membrane's ability to exchange Na⁺ from the inside for K⁺ from the outside; the process is against both Na⁺ and K⁺ concentration gradients. The result is that Na⁺ and K⁺ are no longer passively distributed and the membrane potential is theoretically no longer equal to the equilibrium potential (inside-outside) for each permeating ion, as given by the Nernst equation:

\[ E = \frac{RT}{2F} \ln \frac{[x]_o}{[x]_i} \]

The membrane potential therefore must be calculated using the modified Goldman's equation:

\[ E = -\frac{RT}{F} \ln \frac{P_K[K^+]_i + P_Na[Na^+]_i + P_Cl[Cl^-]_o}{P_K[K^+]_o + P_Na[Na^+]_o + P_Cl[Cl^-]_i} \]

This equation can be simplified when one considers the relative permeabilities \( P_K \), \( P_{Na} \) and \( P_{Cl} \). The red cell membrane is much more permeable to Cl⁻ than it is to either Na⁺ or K⁺ (Tosteson, 1959). The relative permeabilities are
approximately 0.6:1:10^6 for K^+:Na^+:Cl^- respectively (Harris & Maizels, 1951; Harris, 1954; Tosteson, 1956; Glynn, 1957a; Tosteson & Hoffman, 1960). Thus, the contributions of the Na^+ and K^+ permeabilities to the membrane potential are negligible when compared to that of the Cl^-.

The membrane potential can be approximated by considering the chloride ratio alone. The Goldman equation is then reduced to the Nernst equation and the membrane potential becomes in effect a chloride potential, i.e.:

\[ E = -\frac{RT}{F} \ln \frac{[\text{Cl}^-]_o}{[\text{Cl}^-]_i} \]

It should be noted that the membrane potential turns out to be due to the chloride distribution alone only because of the insignificance of the Na^+ and K^+ contributions, and not because of any basic assumption that Cl^- and not Na^+ or K^+ are passively distributed.

As the red cell ages and approaches the end of its life span, its metabolic activity decreases. The gradients of Na^+ and K^+ across the membrane are lower in old cells than in young cells. This may result in redistribution of the Cl^- to a different ratio, and therefore a change in the membrane potential with cell age.
V. PROBLEMS IN DIRECT MEASUREMENT OF MEMBRANE
       POTENTIALS IN RED CELLS

(1) TECHNICAL PROBLEMS

The success of obtaining membrane resting potentials from direct measurements depends mainly on the ability of the cell membrane to seal around the electrode tip immediately after puncture. The use of ultra-fine glass microelectrodes has recently been developed into an extremely sophisticated tool for this purpose. Microelectrodes with tip diameters less than 1 μ have been used successfully in studies of small single cells such as red cells and white cells (Lassen & Sten-Knudsen, 1968; Jay & Burton, 1969; Beckmann et al., 1970) and indeed even isolated mitochondria (Tupper & Tedeschi, 1969), cell organelles with a diameter of only about 5 μ.

Owing to the disc shape of the normal red cell, measurements with microelectrodes are difficult. Although the cell has a diameter of about 8 μ, it is flat, and the thickness is only 1.0 μ and 2.4 μ respectively at the dimple and at the rim. In a cell suspension, a red cell will fall
to the bottom because it has higher specific gravity than the medium, either plasma or isotonic Ringer solution. The cell will land on its flat side at the bottom, which is the stable position. Penetration of the cell membrane is possible only from the side at the rim, without breaking the fine tip of the microelectrode. An approach from the top will provide a larger target but it is impossible to avoid overshooting a 1μ thickness, and the microelectrode will break if it hits the coverslip.

The mechanical fragility of the cell membrane also adds to the technical difficulties. When a microelectrode penetrates the red cell, it does so by "ripping" a hole in the membrane. Supposing the tip diameter of the electrode is only 0.5μ, to the red cell it still represents 20% of its own maximum thickness, and the damage to the membrane as the microelectrode enters the cell may be very extensive. Tension exists in the membrane, and there is a positive pressure inside the cell (Rand & Burton, 1964). Extensive damage to the membrane will allow outward leakage of cytoplasm, and complete destruction of the red cell. This problem is not encountered in studies of larger cells because the membrane damage is relatively speaking, very small and any leakage will be quite localized with respect to the whole cell.
Present day technology has made it possible to make ultra-fine glass microelectrodes for the study of small cells. Manufacturers have claimed that micropipette pullers with good control of current and rate of pull can produce glass micropipettes with tip diameters as small as 0.2\mu.

Glass microelectrodes are usually filled with electrolytic solutions, the most commonly used is KCl at a concentration of 3 molar. The 3 molar concentration provides a solution of sufficiently high conductivity and the choice of KCl over other electrolytes is an attempt to approach the ideal situation where the dissociated cations and anions have equal mobilities. As we have discussed in a previous section, unequal mobilities between cations and anions in a solution can result in a diffusion potential being established, a situation that is undesirable in electrode work.

The mobilities of Na\(^+\), K\(^+\) and Cl\(^-\) ions in solutions at 25\(^\circ\) C are 5.91 \times 10^{-4}, 7.62 \times 10^{-4} and 7.91 \times 10^{-4} \text{ cm}^2/\text{sec/volt} respectively (Moore, 1962, p. 337). It can be seen that the K\(^+\) and Cl\(^-\) ions have almost equal mobilities in KCl solutions while the Na\(^+\) and Cl\(^-\) ions in solutions of NaCl will have quite different mobilities. The ideal microelectrode should also be small in tip
diameter and have low electrode resistance. Since the two are contradictory, some compromise has to be made. Micro-electrode resistance can be minimized by selection of glass stock which controls the geometry of the electrode tip, and by filtration of the electrolyte solution in the filling procedure. These will be discussed in the methods section.

(2) THE LIQUID-JUNCTION POTENTIAL

When an electrolyte-filled microelectrode tip is placed in an environment containing an electrolyte in solution, a "liquid-junction potential" exists across the junction of the two electrolyte solutions. This junction potential is zero if and only if the two solutions are identical in both species and concentration. Liquid-junction potentials can be calculated from the Henderson equation (Henderson, 1908; see Ives & Janz, 1961, p. 54) and is given by the following expression when only univalent ions are involved and there is only one anion and one cation in each solution:

\[ E_J = \frac{RT}{F} \cdot \left[ \frac{U_1 - V_1}{U_1 + V_1} \right] - \left[ \frac{U_2 - V_2}{U_2 + V_2} \right] \cdot \ln \left[ \frac{U_1 + V_1}{U_2 + V_2} \right] \]

In this equation, \( E_J \) is the liquid-junction potential and \( R, T, F \) have their usual significance. \( U \) and
V are the concentration and ionic mobility product terms, for the cation and the anion respectively when all the mobilities are taken as positive. The subscripts 1 and 2 indicate the two different solutions. This equation assumes the continuity of composition across the junction but contains no characteristic of the geometry of the junction. When an electrolyte-filled microelectrode is placed in a solution, the liquid-junction potential has only the effect of off-setting the zero or reference potential. Difficulties arise however in membrane potential measurements when the electrode tip is moved from the extracellular to the intracellular medium. Because of the differences in concentrations and ionic mobilities in the two media, the magnitude and even the polarity of the liquid-junction potential may change as the electrode is advanced through the membrane into the cell, and is no longer just a constant off-set. This change in junction potential appears as part of the measured value and causes an uncertainty in the measurements.

In studies of cells where the transmembrane potential is high, the contribution from this change of junction potential to the measured potential can safely be disregarded when 3M-KCl-filled micropipettes are used (Cole & Moore, 1960). In the present situation with the red cell, the external medium is essentially a NaCl
environment while the internal medium is one of KCl. The cell also contains a high concentration of negatively charged hemoglobin which may cause a reduction of the ionic mobility of the K⁺ ions in the intracellular medium. With a chloride ratio of $[\text{Cl}^-]_o/[\text{Cl}^-]_i = 1.34$ the Nernst equation predicts a membrane potential of only about $-7.5\text{mV}$ at room temperature.

Thus the contribution of the change in liquid-junction potential cannot be disregarded completely without knowing at least its order of magnitude. This has been considered in the case of red cells in serum at $37^\circ \text{C}$, by Lassen and Sten-Knudsen (1968). The authors estimated that even with a six-fold reduction of the mobility of K⁺ ions in the cell due to the presence of the hemoglobin, the change in the liquid-junction potential to be expected as the microelectrode penetrates the membrane is only $\pm 0.3\text{mV}$. Since their experimental error is in the range $0.5-1\text{mV}$, a correction for the liquid-junction potential does not appear to be very important.
VI. METHODS 1 - APPARATUS FOR MEASUREMENT OF POTENTIALS

(1) GLASS MICROPETTE ELECTRODES

The microelectrodes used in this study were glass micropipette electrodes filled with 3M-KCl solution. The stock glass capillaries had 1 mm O.D. and were 6 inches long supplied as special pyrex capillaries 520-119 (Walter A. Carveth Ltd.)\(^1\). Micropipettes were made using a vertical micropipette puller (David Kopf Instruments)\(^2\) which the manufacturer has claimed could consistently produce micropipettes with tip diameters no larger than 1/4\(\mu\). The micropipettes were filled with 3M-KCl. Initially, the micropipettes were filled by low-pressure boiling. Micropipettes were pulled, placed in the holder and immersed in the KCl solution (Figure 2a). The solution, which had been filtered through a 0.22\(\mu\) millipore filter (Millipore Filter Corp.)\(^3\), was heated to 80\(^\circ\)C. The flame was removed

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\(^1\) Walter A. Carveth Ltd., Toronto, Ontario, Canada.
\(^2\) David Kopf Instruments, Tujunga, California, U.S.A.
\(^3\) Millipore Filter Corp., Bedford, Mass., U.S.A.
FIGURE 2

Filling micropipettes with 3M-KCl solution.

(a) by low pressure boiling at 80°C.
(b) by capillary action at room temperature.
and the vacuum gradually turned on so that the solution boiled gently but steadily for at least 3 minutes. The vacuum was released slowly by inserting a hypodermic needle into the connecting rubber tubing, and the micropipettes were filled when the air inside the pipettes rapidly dissolved into the solution. Microelectrodes prepared by this method usually had resistances as high as 50MΩ and tip potentials up to 10 mV. A small portion of the microelectrodes would have tip potentials of about 5 mV, and these had been used successfully in initial measurements (Jay, 1968). Microelectrodes with tip potentials larger than 5mV or with tip potentials which changed when the tip was pressed against the coverslip were not used.

Boiling of the micropipettes in 3M-KCl apparently leaves deposits close to the tip (Lassen & Sten-Knudsen, 1968) which can cause excessive damage to the membrane during penetration. Subsequently, our methods were modified so that the micropipettes were filled without boiling (Figure 2b). The micropipettes were placed in the holder and the tips immersed in 3M-KCl for at least 24 hours. The tips would fill, by capillary action, to the top of the taper. The rest of the pipette was filled using a 30 gauge hypodermic needle and a 1 ml syringe. Long glass capillaries of 50-100μ diameter drawn from the stock glass had also been used in place of the hypodermic needle
especially when capillary action had only filled the micropipettes to the bottom of the taper.

The microelectrodes filled this way without boiling had resistances from 15 to 30 MΩ and many would have tip potentials less than 5mV, the upper limit used for choice of microelectrodes for this study.

Similar glass microelectrodes but with larger tip diameters, from 0.5 to 1μ were used for ground electrodes which serve as reference for measuring the potential differences. These larger tip microelectrodes were lower in resistance by at least one order of magnitude and in effect, served as low resistance grounds which could be placed accurately near the cell being studied.

(2) THE REVERSIBLE SILVER-SILVER CHLORIDE JUNCTION

The 3M-KCl solutions in both the measuring and the ground electrodes had to be connected to the amplifier. For this purpose, reversible silver-silver chloride junctions were used. A reversible junction is one across which no polarization is set up when a small current passes either way, and the energies for charge transfer are the same in both directions. Completely reversible and nonpolarizable liquid-metal junctions exist only in theory. In practice, junctions are considered reversible if the polarization is
beyond the sensitivity of the measuring instrument. The silver-silver chloride junction is one such junction and has been widely used in membrane potential studies.

Figure 3 shows a schematic diagram of the micro-electrode holder (Bioelectric Inst. Inc.)\textsuperscript{4} which contained the Ag-AgCl junction and provided the coupling between the 3M-KCl in the microelectrode and the input of the amplifier. Two types of Ag-AgCl junctions had been used successfully. The first consisted of a coiled silver wire electrolyzed in the dark for 6 hours in a solution of either 0.1M NaCl or 0.1M HCl. The current density used was 500 $\mu$A/cm\textsuperscript{2}.

The second type of junction consisted of a pressed AgCl pellet with a fine silver wire for connection (Bionetics Electrochem Inc.)\textsuperscript{5}. This was soldered on to the microelectrode holder and the solder joint covered with epoxy.

Usually 6 junctions of either type were prepared at the same time (Figure 4). The junctions were equilibrated in 3M-KCl for 20 hours and then their junction potentials were measured with respect to the same reference. Junctions with potentials within 0.1 mV of each other were paired for use in the active and ground electrodes. The 20 hours aging period provided sufficient time for the junction potentials

\textsuperscript{4}Bioelectric Inst., Inc., Hastings-on-Hudson, New York, 10706, U. S. A.

\textsuperscript{5}Bionetics Electrochem, Inc., Santa Ana, California, 92705, U. S. A.
FIGURE 3

Schematic diagram of microelectrode holder.

Two types of reversible junctions are shown:
(i) silver-silver chloride wire junction
(ii) silver chloride pellet junction.
Schematic diagram of apparatus for the preparation of reversible junctions.

(a) Six Ag-AgCl junctions were electrolyzed simultaneously in either 0.1M NaCl or 0.1M KCl solution. A DC current source provided a current density of about 500μa/cm². The procedure was carried out in the dark.

(b) Equilibration of junctions in 3M-KCl solution and measurement of relative junction potentials for matching pairs.
to reach their equilibrium values (Ives & Janz, p. 214, 217), and since the junctions in both the measuring and ground electrodes had the same potential, the measured tip potential would be due to the tip potentials of the glass micro-electrodes alone.

Both types of junctions were equally satisfactory. The electrolyzed junctions were economical but required rechloridizing from time to time. The AgCl pellets were expensive and fragile, but did not require much maintenance. Care must be taken however, in putting the epoxy around the soldered joint so that only AgCl is in contact with the KCl solution.

(3) THE AMPLIFIER-RECORDE SYSTEM

The use of high resistance glass microelectrodes requires the use of an amplifier with high input impedance. The amplifiers used in this study were the DS2C and the NF-1 models (Bioelectric Inst. Inc.)\(^4\). The DS2C model with electrometer tube in the input circuit was first used, but was replaced by the NF-1 model when it became available as an improved version. The NF-1 model used field-effect transistors in the input circuit and had the advantage of not picking up mechanical vibrations and had lower noise level. Otherwise, both amplifiers had basically similar
specifications. Neutralized input capacity was featured in both models. Figure 5 shows the difference in output wave form, from a square-wave input, with and without neutralization of the input capacity due to the high resistance glass microelectrode in the circuit. The input impedance of the amplifier was $10^{12}$ ohms and the grid or leakage current was less than $10^{-12}$ ampere. With neutralization, the amplifier operated at a gain of 2 with maximum negative feedback, to 5, above which oscillations would occur.

The amplifiers also featured built-in microelectrode resistance measuring circuits (Figure 6). It was basically a voltage divider which consisted of the unknown microelectrode resistance $R_e$ and the standard resistor $R_1$. The DC resistance of the microelectrode was measured with a very small current when a voltage of ±1 volt is applied. $R_e$ was calculated from $E$, the voltage drop across itself, knowing the value of the standard resistor $R_1$ and the applied voltage.

The recording system consisted of a Beckman type RP dynograph (Beckman Instruments, Inc.) with 16 chart speeds ranging from 1 mm/min. to 250 mm/sec. and a Tektronix type 564 storage oscilloscope (Tektronix Inc.) with type

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6. Beckman Instruments, Inc., Fullerton, California, U.S.A.
7. Tektronix Inc., Portland, Oregon, U.S.A.
FIGURE 5

Comparison of amplifier output waveforms from a 10mV-60cps square-wave input. Microelectrode resistance 30MΩ.

(a) Without neutralization of input capacitance
(b) At maximum neutralization without oscillation.

Calibration voltages of either 5 or 10 mV were used. Amplifier gain was usually set at 3, 4 or 5.

Grid squares: 10 mV (vertical)
2 msec (horizontal)
gain set at 4
FIGURE 6

Diagram of microelectrode resistance measuring circuit.

The DC resistance of the microelectrode is measured with a very small current of $10^{-9}$ amperes or less. The circuit is basically a voltage divider consisting of the standard $10^9 \Omega$ resistor and the unknown $R_\text{e}$. $R_\text{e}$ is given by the relation:

$$R_\text{e} = \frac{10^9}{V}$$

where $E$ is the measured voltage drop across $R_\text{e}$, in volts. The $10^4 \Omega$ resistor shown in the circuit serves as a convenient ground return. Its value is too small to significantly add to the $10^9 \Omega$ resistance. $V$, the input voltage is $\pm 1$ volt.
2B67 time-base unit. In the initial studies, only the chart recorder was used for recording the data. As the technique became more sophisticated, the storage feature of the oscilloscope was extensively used when the external trigger switch was coupled to the microelectrode advance mechanism.

(4) MICROSCOPE AND MICROMANIPULATORS

A Nikon inverted microscope (Nippon Kogaku Inc.) provided the optics for this study. For micromanipulation, two Leitz mechanical micromanipulators (E. Leitz) and one deFonbrune pneumatic type micromanipulator (Aloe Scientific) were used. Figure 7 shows the microscope and micromanipulators as used in this investigation.

The red cells in a suspension placed in a cavity slide would settle to the bottom and stick to the cover-slip. The cells could then be impaled without having to be held by a second instrument. If a conventional type microscope and the usual hanging drop method were used, this would not be possible.

8 Nippon Kogaku (U. S. A.) Inc., Garden City, N. Y., U. S. A.
9 E. Leitz, Wetzlar, Germany
10 Aloe Scientific, St. Louis, Mo., U. S. A.
FIGURE 7

Apparatus used for micromanipulation and measurement of membrane potentials from red blood cells.
Because of crenation problems which occur when red cells come into contact with glass (Ponder, 1948, p. 40), all slides and cover-slips used were plastic unless otherwise specified. Figure 8 shows a plastic cavity-slide chamber and the relative positions of the optical elements and microelectrodes during measurement.

While initial measurements were successfully obtained using only the Leitz micromanipulators, improvements of techniques led to the coupling of the deFonbrune pneumatic type to the Leitz unit in order to provide rapid advancement of the microelectrode through the cell membrane. Figure 9 shows a schematic diagram of the coupled micromanipulators. The deFonbrune pneumatic one which had very fine movements effected by compression of air bellows was mounted on a Leitz mechanical type which provided sufficient support as well as convenient coarse movements. The rubber air-line between the air bellows for forward movement and the control joy-stick was clamped under the plate of a battery-operated relay. The switch which closed the circuit to activate the relay also triggered the storage oscilloscope, which was set for single-sweep at 20 or 50 msec/cm. The closing of the battery circuit simultaneously started the sweep of the oscilloscope beam and activated the relay, which clamped the air-line, to advance the microelectrode.
FIGURE 8

Schematic diagram showing plastic cavity-slide and relative positions of the cell, microelectrodes and optical elements during measurements.
FIGURE 9

Schematic diagram of the microelectrode rapid advance mechanism.
By adjustment of the position of the rubber air-line at the relay, an instantaneous $2\mu$ advance of the microelectrode without backlash was possible. The opening of the battery circuit retracted the microelectrode.
VII. METHOD 2 - PREPARATION OF BLOOD SAMPLES

(1) PREPARATION OF MIXED-AGE CELL POPULATIONS

In studies of mixed-age populations, red cells were taken either from finger prick or from venipuncture. The cells were suspended in modified isotonic buffered Ringer solution (see Appendix A), at 23(±1)°C. The final concentrations of the major ions in the solution were 110 mEq/L Na⁺, 4.5 mEq/L K⁺ and 140 mEq/L Cl⁻. The osmotic pressure was 310(±5) mOsm, and pH was buffered at 7.40 (±0.02) with HCl-THAM (Tris-hydroxymethyl aminomethane) buffer. The resultant Ringer solution was similar to plasma in osmotic pressure, pH, and concentrations of Na⁺ and K⁺, with slightly higher Cl⁻ which raised the osmotic pressure sufficiently in absence of the various plasma proteins.

A finger prick was made with a blood lancet. About 2 cubic millimeters of blood was drawn into a 1 ml syringe. The syringe was filled with the prepared Ringer solution and the red cells were dispersed by repeatedly
inverting the syringe. A drop of the cell suspension was placed in a plastic cavity-slide, the bottom of which was made of a plastic coverslip. Ringer solution was added to the cavity until the surface was flush with the top of the slide. Within a few minutes, the cells would all settle to the bottom and would be about half a cell diameter apart without overlap. The microelectrodes were lowered into the solution and measurements were made.

In sampling by venipuncture, 10 ml of venous blood were drawn and placed into a vacutainer containing sodium heparin. For studies of populations of unwashed cells, the usual procedure of suspending cells in Ringer solution was carried out without any further preparation.

The choice of either method for sampling was mainly dependent on the subject. There was no sign of any difference between cells obtained by the two methods from the same subject.

(2) SEPARATION OF YOUNG AND OLD CELLS ACCORDING TO DENSITY BY CENTRIFUGATION

In the study of relation between the membrane potential and the physiological age of the red cell, all blood samples were taken by venipuncture. Ten ml of venous blood were drawn and immediately heparinized in a
vacutainer. The sample was centrifuged at 1500 g for 10 minutes in a 10°C cold room. A clinical centrifuge (International Equipment Co.)\textsuperscript{11} with 15 ml graduated glass tubes were used. The plasma was removed and replaced with an equal volume of the prepared Ringer solution. The packed cells were completely dispersed by shaking and centrifugation was repeated. After three washes with the Ringer solution, the cells were resuspended to make up the original hematocrit. The washing procedure had completely removed the plasma which contained fibrinogen, the substance associated with rouleaux and aggregate formation. The cells would no longer adhere to each other, and the sample was ready for the separation procedure.

Eight microhematocrit tubes, 1.2 mm I.D., 75 mm long were loaded to within 2 mm of the top with the washed cell suspension, and centrifuged at 11,000 g for 30 minutes in the cold room. An Adams Autocrit centrifuge model CT-2905 (Clay-Adams, Inc.)\textsuperscript{12} was used. The cells settled and packed according to density with the top of the column being rich in young cells and the bottom consisting mainly of old cells. Figure 10 shows a packed cell column after

\textsuperscript{11} International Equipment Co., Needham Heights, Mass., U.S.A.

\textsuperscript{12} Clay-Adams, Inc., New York, N.Y. 10010, U.S.A.
A packed cell column in a microhematocrit tube.

Upon centrifugation at 11,000g for 30 minutes, red cells packed forming a continuous density gradient. The young cells with lower density were mainly on top while the older cells with higher density were mainly at the bottom.

The diagram shows the four age groups studied. The cell profiles with different shadings represent the relative cell densities or age, which increases with depth in the tube.
ISOTONIC RINGER SOLUTION

MICROHEMATOCRIT TUBE

CELL FRACTION

RELATIVE AGE

TOP 10% { YOUNG CELLS

4th 10% { YOUNG MIDDLE AGE

7th 10% { OLD MIDDLE AGE

BOTTOM 10% { OLD CELLS

PLASTICINE SEAL
centrifugation, and the relative age groups studied. The cell column was marked into ten equal parts along its length representing ten mean age groups. Each age group consisted of 10% of the total cell column. Four groups, the top, fourth, seventh and bottom 10%, were studied. Since cell density and cell age are approximately linearly related, the four groups had mean cell ages of very approximately 6, 42, 78 and 114 days. In this study, the four groups will only be referred to as young, young middle age, old middle age and old cells respectively, to indicate only the relative ages of the cells according to density.

Sampling from any age group was done by accurately cutting the capillary tube at the bottom of the required 10%. All of the 10% was then drawn into a 1 ml syringe. The cells were completely dispersed in Ringer solution and prepared for study in a cavity-slide as previously described.

(3) PREPARATION OF SEPARATED AND RE-MIXED POPULATIONS

The procedure of separating young and old cells subjected the red cells to repeated washing in Ringer solution, to cold temperature, and to ultracentrifugation at very high g. It was quite possible that any or all of these would cause irreversible changes in the cell, and alter the membrane potential. To see whether the procedure
alone had altered the membrane potential, normal populations of cells which had gone through the separating procedure were thoroughly re-mixed and studied.

All the cells in a packed cell column from a microhematocrit tube were drawn into a syringe and completely dispersed in Ringer solution. After appropriate dilution, the cells were studied and the membrane potentials were compared to those obtained from cells which had not gone through the separating procedure.

(4) CRENATED RED CELLS

Red cells quite often "crenate" when they come into contact with glass (Ponder, 1948, p.40). The reason why they do is not completely clear although the effect of charge on surfaces is certainly connected with it. The crenated cells are no longer biconcave in shape, and the membranes show "protrusions" or "bumps". This phenomenon was seen in our experiments when glass cover-slips were substituted for the plastic ones. These crenations were different from hypertonic crenations since the extracellular medium was still at isotonic strength, and the crenations were readily reversible. When the slide was turned upside down and the cells freed from the glass surface, the crenated cells returned to normal biconcave shape within seconds.
Although nothing was known about the cell interior under these conditions, a measure of the membrane potential in the crenated cells might point the direction in which we could speculate about these changes.

For our experiments, samples were prepared as before, but glass cover-slips were used. Normal, mixed-age unwashed cells were studied. The slides were allowed to stand for about 5 minutes and the cells settled on the cover-slips. Most of the cells in contact with the glass cover-slip were crenated with a few per cent of the population being still biconcave in shape. While Ponder (1948) reported different stages of crenation, all our cells which were crenated appeared to be quite uniform in shape throughout the sample. These cells were studied, and a few of those which remained biconcave were studied along with the crenated ones.

(5) AGGREGATES OF RED CELLS

When red cells are suspended in plasma or some other isotonic medium containing "long-chain" molecules, they stick together in an orderly fashion like stacks of coins. These are called "rouleaux". If the sample is undisturbed and the hematocrit is high, eventually the rouleaux will clump together to form aggregates of red cells.
If a microelectrode is advanced into an aggregate, although the cell being studied could not be observed, the microelectrode would inevitably penetrate one cell or another, and the change in potential as the microelectrode penetrated the membrane could be measured.

A cavity-slide was filled with plasma and a drop of whole blood was added and mixed. A cover-slip was placed over the cavity and the sample was observed through the microscope. After about 10 minutes, the cells had formed large "aggregates of rouleaux". The cover-slip was removed and measurements were made.

(6) WHITE CELLS

White cells or leukocytes are normally present in blood at approximately 7,000 per cubic millimeter. This is about one white cell to every 600 red cells in a sample of blood. Despite their relatively small number, they were seen in the samples occasionally during our experiments. Although they are completely different from red cells, it was felt that some measurements of their membrane potentials would be of additional interest. Membrane potentials in 18 white cells only were measured with microelectrodes, and
no attempt was made to classify the individual white cells
(see Wintrobe, 1961, p. 247).

(7) SEMIPERMEABLE MICROCAPSULES CONTAINING HEMOGLOBIN

A semipermeable microcapsule containing hemoglo-
bin, placed in Ringer solution reaches a Donnan equilibrium
across the membrane. If the membrane is freely permeable
to Na\(^+\) and Cl\(^-\) ions, but is impermeable to the hemoglobin
molecule then, knowing the concentration of hemoglobin
inside, the Donnan potential across the membrane can be
calculated.

Semipermeable microcapsules containing hemoglobin
were prepared according to methods first developed by
Chang (Chang et al., 1966) and used in previous studies on
properties of microcapsules (Jay & Edwards, 1968; Jay &
Sivertz, 1969). Resultant microcapsules had nylon membranes
which were impermeable to hemoglobin but were freely permeable
to electrolytes. The final concentration of hemoglobin in
the microcapsules was about 20% of that in normal red cells.
The microcapsules, 20 to 500\(\mu\) in diameter, were suspended
in Ringer solution as used in red cell experiments.

The Donnan potential across the membrane was
calculated to be less than \(-1\) mV (See Appendix B). This
potential was measured directly by microelectrode punctures
and compared to the calculated value. The difference would give some estimate of the change in the liquid-junction potential due to the presence of hemoglobin.
VIII. METHODS 3 - MEASUREMENT OF HUMAN
RED CELL MEMBRANE POTENTIALS

(1) CALIBRATION OF MEASURING SYSTEM

A cavity-slide was filled with Ringer solution and placed on the microscope stage. The tips of the measuring and the ground electrodes were lowered into the solution and observed through the microscope. The measuring system was connected as shown in Figure 11. With the reference electrode switched to ground, the zero-line was set. The reference was then connected to the calibrator output of the oscilloscope. A 60 cps, 5 or 10mV square-wave was used as the calibrating voltage and the gain of the amplifier was set at either 3, 4 or 5 depending on the amount of neutralization required for obtaining a satisfactory rise-time.* The amount of neutralization required varied from one electrode to another and the procedure was repeated whenever electrodes were

*Rise-time taken as time required for an amplifier output to rise from 10% to 90% of maximum in response to an input step function.
FIGURE 11

Schematic diagram of the measuring system.
replaced. In all the experiments, the gain and the neutralization were appropriately adjusted so that the rise-time was always better than 0.5 msec.

A ±10mV DC source provided by a mercury cell had also been used for calibration purposes, in addition to the square-wave from the oscilloscope. Both sources gave the same calibration. The calibration was checked from time to time during the experiments to ensure that it had not changed.

(2) MEASUREMENT OF TIP POTENTIAL AND MICROELECTRODE RESISTANCE

The selection of satisfactory microelectrodes for measurements required meeting three criteria. Firstly, the microelectrode was pressed lightly against the cover-slip. If the zero-line showed any shift at all, the electrode was discarded. The electrodes were mounted at about 20 degrees to the horizontal, and pressing lightly against the cover-slip usually did not damage the tip. A shift in the zero-line would represent a change in the tip potential due to mechanical stresses on the microelectrode tip alone. Such electrodes, which were sensitive to mechanical stresses applied at the tip might cause artifacts during cell punctures, and were not used.
The second criterion to be met was an upper limit in steady tip potential. The tip potential was measured by the shift in the reference or zero-line when the input of the amplifier was shorted to ground. The reference electrode was connected to ground and the zero-line noted. The input to the amplifier was shorted by closing the switch S as shown in figure 11, and the shift in the zero-line was measured as the tip potential. Microelectrodes with unsteady tip potentials or tip potentials larger than 5 mV were discarded. The microelectrodes used in the study had tip potentials between 2 and 5 mV.

Finally, the resistance of the microelectrode was measured, using the voltage divider previously described. The microelectrodes selected for measurements had resistances of less than 50 MΩ. Microelectrodes with resistances higher than 50 MΩ were usually quite noisy electrically. Since satisfactory microelectrodes with lower resistances had been made, the upper limit was arbitrarily set at 50 MΩ.

(3) MEMBRANE POTENTIAL MEASUREMENTS FROM SINGLE RED CELLS

A single red cell was observed through the microscope. The microelectrodes were placed so that the tip of the ground electrode was about one cell-diameter distant from the cell and that of the measuring electrode
was within one micron from the rim of the cell. The microscope was sharply focused on the cell and the height of the measuring microelectrode was adjusted, by the fine movement of the micromanipulator, so that the tip came into focus, and was therefore at the same level as the rim of the cell. The switch, which operated the relay, was closed. This simultaneously started the oscilloscope beam to sweep and advanced the microelectrode into the cell. Figure 12 shows photomicrographs of single red cells being penetrated by microelectrodes during measurements.

Following penetration by the microelectrode, the cell usually would hemolyze within a few seconds, even if the microelectrode was not retracted. The cell "ghost" under phase-optics appeared as a dark, perfectly circular shadow of about 6μ diameter. In almost all of the measurements made, the microelectrode was retracted after about 1 sec., in order that the abrupt return of the deflection to the reference zero be observed on the chart recorder. When the microelectrode was retracted, the cell almost invariably hemolyzed. It was extremely rare that hemolysis did not occur immediately following retraction.

In preliminary studies where the storage feature of the oscilloscope was not available, the magnitude of the potential was measured exclusively from the chart records.
FIGURE 12

Photomicrographs showing red cells being impaled by microelectrodes. The ground electrodes are also shown.

(12f) shows the deformation of the cell if the microelectrode was not able to penetrate the cell membrane. This happened frequently when slow manual advance of the microelectrode was used.

(12c) shows the same cell successfully penetrated by rapid-advance of the microelectrode.
In addition, the microelectrode rapid-advance mechanism was not used and cell penetration was done manually. Since potentials which remained reasonably constant for as long as 30 seconds before hemolysis occurred had been recorded, the criterion for a "good" measurement was arbitrarily set such that the constant period should be at least 5 seconds following penetration. The strict criterion was an attempt to eliminate all measurements in which any possible damage to the membrane might have occurred.

With this strict criterion, satisfactory measurements were extremely hard to obtain. It was also felt that a bias might have been created resulting in preferential recording of measurements which are larger and likely to remain steady for longer. With the improvement of the microelectrode rapid-advance mechanism, we were able to see the deflection immediately after penetration on the stored trace in the oscilloscope. The slow response of the chart recorder pen no longer presented a problem. Lassen et al. (1969), in the study of membrane potentials in Ehrlich ascites cells, have reported that a higher initial transient potential of several milliseconds duration, rather than the lower constant value which follows, appears to agree better with the predicted value of the membrane potential for that cell.
In the red cell, no transient potentials were observed. Nevertheless, it was decided that the first 100 msec would have given the true measurement and the requirement that hemolysis did not occur within 5 seconds was not justified and might have introduced bias. Subsequent measurements used the storage feature of the oscilloscope exclusively. Satisfactory measurements were taken as those which did not decrease by more than 10% over the first 20 msec. The magnitude of the membrane potential was measured directly from the oscilloscope. Deflections which stayed reasonably constant over seconds could also be measured from the chart recordings which also show the deflection returning to zero upon retraction of the microelectrode.

(4) MEASUREMENTS FROM CRENATED RED CELLS, AGGREGATES, WHITE CELLS AND MICROCAPSULES CONTAINING HEMOGLOBIN

Measurements from crenated red cells and from white cells required slight modification of technique. The crenated cells no longer adhered to the cover-slip and penetration by microelectrodes could not be effected. A glass rod, about 10μ in diameter with a flat tip was used to stop the cell from sliding during penetration. A de Fonbrune microforge (Aloe Scientific) was used to fabricate
the glass micro-rod. A micropipette was heated at the tip to fuse the open end. The end was then broken off under tension to give a flat tip, which was slightly fire polished.

Penetration of crenated cells by microelectrodes did not cause hemolysis until withdrawal of the microelectrode. A few crenated cells did not hemolyze even upon retraction of the microelectrode and a repeated measurement from the same cell was possible. Comparatively, measurements from crenated cells were easier to obtain. Although the diameters were only about 6 μ in diameter, the cells were rounded which made for larger effective targets.

White cells were similarly studied. They provided even larger targets of about 10 μ in diameter. Penetration by microelectrodes were not difficult and the cells usually remained intact during measurements. In measurements of both crenated red cells and white cells, the microelectrode tip was easily freed from the cells upon retraction.

Measurements in red cell aggregates were done simply by advancing the microelectrode slowly into an aggregate of about 100 cells. It was not necessary to hold the aggregate by any means. The method did not provide any information as to which cell was being studied, nor did it indicate at any time whether the microelectrode was in a cell or in the intercellular space.
The large size of the semipermeable microcapsules containing hemoglobin made them extremely easy to study. Microelectrodes used had tip diameters of about 0.5μ and microelectrode resistances were 5 to 15 MΩ. Slightly larger microelectrodes were necessary due to the comparatively high mechanical strength of the nylon membranes. As a result, all microelectrodes prepared by the usual method were useful for this purpose. The finer ones would have their tips broken at the first penetration resulting in a 0.5μ tip. Retraction of the microelectrode did not free the microcapsule from its tip, and additional modification to the technique was required. A suction-pipette was used to hold the microcapsule, for impalement by the microelectrode. The pipette had a 10μ tip which was flat and fire-polished. Suction was controlled by a syringe connected to the pipette. Both the pipette and the syringe were filled with Ringer solution which was electrically grounded. A similar technique has been used in the study of the microcapsule membrane resistance (Jay & Sivertz, 1969). With the suction holding the microcapsule, it could be easily impaled, and retraction of the microelectrode presented no problem. Upon retraction of the microelectrode, the microcapsules ruptured, probably due to the very high internal pressure (Jay & Edwards, 1968).
The measurements from crenated red cells, aggregates, white cells and microcapsules were made before the design of our rapid-advance mechanism. Microelectrode advance was effected manually through the fine control of the micro-manipulator, and measurements were recorded on the Beckman chart recorder.
IX. RESULTS AND DISCUSSION

(1) INITIAL MEASUREMENTS

a. Results

Initial results were obtained by slowly advancing a microelectrode into cells and observing the deflection. The criterion for an acceptable measurement was that the deflection should remain reasonably constant for at least 5 seconds. Twenty recordings of steady membrane potentials were obtained. Red cells from two subjects were used. Figure 13 shows the recording obtained from one measurement. It is typical of the chart recordings, except that it has the longest constant period observed. A sharp deflection occurred immediately following the penetration of the membrane by the microelectrode. The deflection remained quite constant and returned to zero upon retraction of the microelectrode. The values of the steady potential ranged from -6.4 mV to -9.6 mV, with a mean value of -8.0 ± 0.9 (S.D.) mV for 20 measurements.

NOTE: All results presented in this chapter are tabulated in detail in Appendix C.
FIGURE 13

Chart recording of membrane potential obtained from one measurement. This is typical of the recordings obtained using the manual micro-electrode advance method during the preliminary investigation. The scale shows 4mV vertical and 20 sec. horizontal.

FIGURE 14

Histogram of measured membrane potentials obtained in the preliminary study. Population of 20 cells has a mean value of \(-8.0\pm0.9(S.D)\) mV.
Figure 14 shows a histogram of the measured membrane potentials.

b. Discussion

In the beginning of our study, there were no guide-lines as to what constituted an acceptable measurement. It was observed that a sharp deflection occurred when the microelectrode entered the cell. In many penetrations, the cells hemolysed and the deflections drifted rapidly back to the zero-line. Thus, a deflection which decayed rapidly was taken to indicate a damaged cell, and therefore discarded as being unacceptable. Steady potentials which were reasonably constant for over 5 seconds had been recorded, and several measurements gave steady potentials which lasted for over one minute. On this basis, a constant period of at least 5 seconds following penetration was arbitrarily set as the measurement criterion. This obviously would have eliminated all cases in which excessive membrane damage occurred.

The strict criterion of measurement was necessary partly because of the slow response of the chart recorder and partly because of the slow manual microelectrode advance technique used during the early stages of our study. While it clearly eliminated all but the very best of measurements, it very possibly could have introduced bias in the mean
value of the membrane potentials in a population of cells. Attempts were made on numerous cells before the 20 successful measurements were obtained. The mean value of -8.0 mV is in very good agreement with the theoretical value of the red cell membrane potential calculated from the Cl⁻ ratio. However, since a large number of cells with smaller deflections which could not satisfy the criterion had been discarded, and since almost all the deflections of magnitudes over -6 mV were steady potentials, it was felt that the possible bias would be towards a higher mean value. It was possible that the cells with higher membrane potentials could sustain membrane damage better than the rest, perhaps because of lower membrane stiffness and higher metabolic activity.

Thus, it was necessary to improve the method of microelectrode advance to allow rapid recording so that the criterion could be relaxed and the possible bias hopefully removed.

The resistance of the red cell membrane was approximately measured in several cells. The resistances were measured as the increase in resistance when the microelectrode entered the cell. The maximum resistance recorded was 6MΩ, a value which is in agreement with those reported by Lassen & Sten-Knudsen (1968).
(2) MEMBRANE POTENTIALS IN MIXED-AGE RED CELL POPULATIONS

a. Results

Figure 15 shows 8 typical examples of stored traces on the oscilloscope obtained from measurements using the improved techniques of rapid microelectrode advance and recording. A sharp deflection occurred as the microelectrode entered the cell interior in each case. The time-lag from when the oscilloscope beam started to sweep, to when the deflection occurred was usually between 20 to 100 msec. The time-lag and its variation depended on several factors. The microelectrode advance mechanism using the compression of air bellows was a mechanical device with an obvious time delay. The initial position of the microelectrode tip relative to the cell might cause some variation in the time-lag. The time-lag would be larger if the microelectrode was advanced from farther off. Finally, the elasticity of the cell membrane might cause some delay since the membrane had to be pushed in by a certain amount before it yielded and allowed the microelectrode to penetrate. This might vary slightly from one measurement to another depending on the actual conditions of measurement.

Figure 16 shows a histogram of the membrane potentials obtained for "unwashed", mixed-age populations of red cells. A total of 46 measurements were recorded. The
FIGURE 15

Typical examples of stored traces on the oscilloscope obtained from measurements using the improved technique of rapid microelectrode advance.

Grid squares: 2.5 mV calibrated (vertical) 20 msec (horizontal)
Histogram of membrane potentials obtained from "unwashed" mixed-age red cell populations. Mixed darkening of cell profiles only represents mixed cell density within the population and does not indicate relative density of individual cells studied.
MIXED AGE CELLS - UNWASHED
-5.6 ± 1.6 (S.D.) mV  N = 46
MIXED AGE CELLS - UNWASHED

-5.6 ± 1.6 (S.D.) mV  N = 46
cells represented 4 blood samples taken from 3 subjects. The potentials ranged from -2.2 mV to -9.0 mV. The total population had a mean value of -5.6 ± 1.6 (S.D.) mV. The mean values for cells from the 3 different subjects were -5.1, -5.4 and -6.7 mV respectively, a range of 1.6 mV. The two samples taken from the same subject had mean values of -4.9 and -5.6 mV, a difference of 0.7 mV.

Figure 17 shows a histogram of the membrane potentials obtained for cell populations which had gone through the washing and density or age separation procedures and had been remixed. A total of 72 measurements were obtained from cells of 5 samples taken from 2 subjects. The total population had a mean value of -5.2 ± 1.8 (S.D.) mV. The mean values for cells from the two different subjects were -4.4 mV and -6.0 mV, a difference of 1.6 mV. The mean values of different samples from the same subject were -4.1, -4.6 and -4.7 mV for one subject and -5.2 and -6.3 mV for the other. These represented maximum differences between mean values of 0.6 mV and 1.1 mV within the two subjects respectively.

b. Discussion: The Measurements

Even with the improved method, measurements were difficult to obtain. Three distinct types of recordings were observed on the storage oscilloscope. A majority of experiments gave no observable deflection at all, as the
FIGURE 17

Histogram of membrane potentials obtained from "washed" mixed-age red cell populations. Population has cells of mixed densities.
MIXED AGE CELLS - WASHED

-5.2 ± 1.8 (S.D.) mV  N = 72
MIXED AGE CELLS - WASHED

-5.2 ± 1.8 (S.D.) mV  N = 72
microelectrode was advanced. In most of these cases the cell remained intact even after retraction of the microelectrode. These were cases where the microelectrode had obviously missed the cells or at least had not punctured the membranes and were not included in our final results. The second type of recordings showed sharp deflections which occurred when the microelectrode was advanced, but which decreased to zero within several milliseconds. These recordings were discarded for obvious reasons. There were probably excessive damage to the membrane and the cells were totally destroyed upon penetration. The deflections might not have given the true values of the membrane potential. The final type of recordings were those which gave reasonably constant deflections for about 100 msec. following microelectrode advance. Examples are shown in Figure 15. Deflections usually fall clearly into either category two or three. Thus, it was usually not necessary to apply the criterion, where the deflection should not decrease by more than 10 per cent within 20 msec., to determine whether a recording was acceptable. Figure 15h shows one of the rare cases. This particular measurement was accepted. Very few deflections occurred in the 0 to -1 mV range in the experiments, and the few that did promptly decreased to zero.

Thus, the improved method of measurement had provided results, probably without bias. The normal
distributions of the populations indicated this. The lower mean values obtained compared to that of the initial experiments showed that indeed, the previously-used strict measurement criterion had introduced some bias in the choice of measurement. Although the measurements did give an idea of the membrane potentials in the human red cells, the true mean value for the population was probably lower than what was obtained.

For comparison, the results of Lassen & Sten-Knudsen (1968) are shown in Figure 18. No statistical evaluation was given the original data. The histogram shown here was prepared from data taken from figure 7 of their publication. The histogram shows a distribution of potentials with a mean value of about -5 mV. The standard deviation is larger than those in our experiments.

While Lassen & Sten-Knudsen consider the higher values of about -14 mV to be the true membrane potential of the red cells and that lower values resulted from cell damage, we think that a cell population has a wide range of membrane potentials, and it is the mean value that should be compared with that calculated from the Nernst equation.

c. Discussion: The Significance of the Mean Values

Both histograms of the unwashed and washed cell populations approximate normal distributions. The mean value of such a population corresponds to the membrane
FIGURE 18

Histogram of membrane potentials obtained from red cells suspended in serum. Data taken from Figure 7 of publication by Lassen and Sten-Knudsen (1968). Mean of population about -5mV.
potential that would be calculated from the chloride ratio using the Nernst equation. If the red cell membrane potential is a chloride potential and that the Nernst equation does correctly predict its value, then the two should be identical. Although membrane potential is a logarithmic function of the chloride ratio, the relation between the two is approximately linear when the Cl⁻ ratio ranges only from 1.0 to 2.0. The mean membrane potential therefore is approximately equal to the membrane potential calculated from the mean chloride ratio.

Table 1 gives the chloride distributions and ratios report by several investigators. The chloride ratios ranged from 1.15 to 1.51. Except for the two extremes, the values compare very well with each other. The mean value is 1.34 regardless of whether the two extreme values are included or not. A chloride ratio of 1.34 predicts a membrane potential of -7.5 mV. The mean values -5.6 mV and -5.2 mV of the two cell populations are low in comparison. Moreover, our Ringer solution had higher chloride concentration than that reported for plasma and therefore the predicted value would be even higher. The discrepancy will be discussed and accounted for in the sections to follow, when the concept of "bound water" and the problem of liquid-junction potential are considered.

The importance of comparing results from washed and unwashed red cell populations has been discussed earlier.
Table 1. Chloride Distribution and Ratio
In Human Red Cells

<table>
<thead>
<tr>
<th>Source</th>
<th>$[\text{Cl}^-]_o$</th>
<th>$[\text{Cl}^-]_i$</th>
<th>$[\text{Cl}^-]_o/[\text{Cl}^-]_i$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hastings et al., (1928)</td>
<td>--</td>
<td>--</td>
<td>1.15</td>
</tr>
<tr>
<td>Maizels, (1936)</td>
<td>110</td>
<td>73</td>
<td>1.51</td>
</tr>
<tr>
<td>Snyder &amp; Katzenelbogen, (1942)</td>
<td>*</td>
<td>113.1</td>
<td>1.27</td>
</tr>
<tr>
<td></td>
<td>**</td>
<td>113.1</td>
<td>1.38</td>
</tr>
<tr>
<td>Bernstein, (1954)</td>
<td>112</td>
<td>78</td>
<td>1.44</td>
</tr>
<tr>
<td>Keital et al., (1955)</td>
<td>M</td>
<td>112.8</td>
<td>1.31</td>
</tr>
<tr>
<td></td>
<td>F</td>
<td>114.5</td>
<td>1.32</td>
</tr>
</tbody>
</table>

Concentrations $[\text{Cl}^-]_o$ are in mEq/L plasma H$_2$O, and $[\text{Cl}^-]_i$ are in mEq/L cell H$_2$O. Where the original data have been given in other units, appropriate conversions have been carried out.

* Values calculated from original data assuming 91% H$_2$O in plasma and 66% H$_2$O in cells (Keital et al., 1955).

** Values calculated from original data assuming 91% H$_2$O in plasma (Keital et al., 1955) and 71.5% H$_2$O in cells (Gary-Bobo & Solomon, 1960).

M = male
F = female
Since our study of the relation between membrane potential and cellular age required washing and centrifugation of the cells, it was important to know whether the procedure had caused any change to the cells. The washed cell population studied had gone through the complete procedure of washing and age separation by centrifugation and had been remixed. Although the mean values of the unwashed and washed population differed by 0.4 mV, because of the large standard deviations, the two populations are statistically not different. A t-test of significance for the difference between the two populations resulted in $0.2 > p > 0.1$. The difference of 0.4 mV between these mean values represented a less than 2 per cent difference in the chloride ratio.

The mean values of normal cell populations varied from one subject to another by as much as 1.6 mV and from one sample to another, taken from the same subject, by as much as 1.1 mV. These mean values were obtained from smaller cell populations of 7 to 37 cells however, and the mean values in some cases may not be accurate representatives of the populations. Still, the possibilities of some variation from subject to subject and within the same subject must not be disregarded since biochemical analyses have also reported corresponding variations in the chloride ratio. A difference of 1 mV in the membrane potential could result from a difference of about 0.05 in the chloride ratio.

Since microelectrodes of resistances ranging from
15 to 50 MΩ were used in our experiments, it was important to know whether the measurements were dependent on the microelectrode resistance. Figure 19 shows a plot of the population means against the microelectrode resistances. There is no significant correlation between the two. The graph also shows the variations from one subject to another and also from one sample to another within the same subject. Some variation in the mean values shown in Figure 19 is probably due to the small number of cells in the populations. They ranged from 6 to 15 cells each.

d. Discussion: The Significance of the Bound Water Concept

The figures in Table 1 show that although the chloride concentrations in plasma reported by various investigators fall within the narrow range of 110 to 114.5 mEq/L plasma water, the chloride concentrations in cells have a much wider range. The figures reported by Hastings et al., (1928) and Maizels (1936) represent opposite extremes of the chloride ratio. Even discounting these and considering only the more recently reported data which appear to be much more consistent, the chloride concentration in red cells still has a range of from 78 to 88.7 mEq/L cell water. With a chloride concentration of 140 mEq/L water in the Ringer solution which we used in the experiments, the transmembrane chloride ratio ranged from 1.58 to 1.80, assuming that the intra-cellular chloride concentration had not changed when
FIGURE 19

Non-dependence of membrane potential on the microelectrode resistance.

Plot of mean membrane potentials of cell populations against resistances of microelectrodes used. The broken lines join populations from the same subjects. Number of cells in the populations ranged from 6 to 15 cells. Standard deviations in the populations range from 0.97 to 2.27mV, two examples of ±1 S.D. are shown.
Ringer solution was substituted for plasma. This chloride ratio range predicts a membrane potential range of -11.6 to -14.9 mV. While the maximum membrane potential measured was -12.8 mV, the means for the populations were only about -5.6 mV. At first sight, it seemed to be much below the expected range. This is not true however, when one takes into account the amount of bound water in the red cell.

The concept of bound water in biological systems has been quite well accepted recently. Microelectrode studies of the striated muscle fibres of the giant barnacle show that the bound portion of the fibre water amounts to 40 to 50 per cent (McLaughlin & Hinke, 1966; Gayton & Hinke, 1970) and in fact, if there were no binding of chloride and if the chloride were passively distributed, the bound water would have been as high as 65 per cent. In the human red cell, 20 per cent of the total cell water has been reported to be osmotically inactive (Savitz et al., 1964; Gary-Bobo & Solomon, 1968). The binding of water on the surface of the hemoglobin molecule observed by Perutz (1946) and Drabkin (1950) has for many years been considered responsible for the anomalous behaviour of cell water. The amount of bound water observed agrees well with the reported value of 20 per cent in the cell. Contrary to his earlier conclusion that 20 per cent of the cell water was osmotically inactive, Solomon (1971) in a very recent article has explained the apparent anomalous behaviour in
terms of change in the hemoglobin net charge as the cell volume is changed. The result is that Cl\(^-\) ions are redistributed to maintain electric neutrality within the cell. While this is probably a valid explanation, and does account for the discrepancy of 20 per cent in the experiments on osmotic behaviour as not being due to bound water, it does not rule out the possibility of the existence of bound water as observed by Perutz and Drabkin.

The hemoglobin molecule at physiologic pH is negatively charged, thus it is possible that Cl\(^-\) is repelled by the hemoglobin, and the water on the surface of the hemoglobin molecule is not available to chloride. Supposing that this results in 20 per cent of the water being unavailable to the chloride then the "effective" intracellular chloride concentration is actually higher than that obtained by biochemical analysis. The measured value will only be 0.8 of the "effective", i.e. the "effective" concentration which the microelectrode sees as it penetrates the membrane, is actually 1.25 times the measured value.

Figure 20 shows the relations between the "effective" chloride ratio and the "measured" chloride ratio when 0, 10, 20 and 30 per cent of the cell water is bound. When the cell water is bound to 20 per cent, the "effective" chloride ratio is unity when the measured value is 1.25. Taking this into account the "effective" intracellular chloride concentration range is actually 97.5 to
FIGURE 20

Relation between the "effective" chloride ratio which the microelectrode sees as it goes through the membrane, and the biochemically "measured" chloride ratio. The lines represent the relations when 0\%, 10\%, 20\% and 30\% of the cell water is bound. In the human red cell, 20\% of the cell water is bound. Note that at 20\%, the "effective" chloride ratio is unity when the "measured" ratio is about 1.25.
111.0 mEq/L cell water.

Figure 21 shows the relation between the membrane potential, as calculated by the Nernst equation, and the "measured" chloride ratio, if all the cell water serves as solvent for chloride, and if 20 per cent of the cell water is bound.

With a concentration of 140 mEq/L water in the Ringer solution, and 20 per cent of the intracellular water being bound, the effective chloride ratio range becomes 1.26 to 1.44. The corresponding range of membrane potential is -5.8 mV to -9.3 mV. Our mean values of $-5.6 \pm 1.6$ (S.D.) mV for the unwashed cells is just outside this range. If the extreme values of chloride ratios reported are included, our mean value actually would fall within the expected range.

The mean value of $-5.6$ mV shows that the "effective" intracellular chloride concentration was 112 mEq/L cell H$_2$O. Biochemical analysis would have measured it as 90 mEq/L cell H$_2$O, if 20 per cent of the water in the intact cell was bound. This is in good agreement with reported figures. If the bound water concept is completely wrong, then the high "effective" chloride concentration inside the cell, which the microelectrodes reported must imply that there had been a chloride shift when the cells were placed in the Ringer solution. The chloride concentration in normal plasma is
Relation between membrane potential as calculated by the Nernst equation and the measured chloride ratio. The chloride ratio range shown (dotted area) on the graph represents the range calculated using the reported range of $[\text{Cl}^-]_i = 78$ to $89$ mEq/L cell water, and $[\text{Cl}^-]_o = 140$ mEq/L as in our Ringer solution. The chloride range of 1.58 to 1.80 predicts a membrane potential range of $-11.6$ to $-14.9$ mV if all the cell water serves as solvent for chloride, but only $-5.8$ to $-9.3$ mV when we consider the water to be 20% bound.
about 114 mEq/L plasma H₂O, while that in the Ringer used was 140 mEq/L. It has been reported that the chloride ion ratio in the red cell is approximately inversely proportional to the hydrogen ion ratio (Harris & Maizels, 1952). Since the pH of the Ringer solution was identical to that in normal plasma, it is possible that the chloride would shift to maintain the original ratio. Thus a higher internal chloride than normally reported could result, and the possible shift could have raised the concentration to about 112 mEq/L cell H₂O. To see whether there was any significant Cl⁻ shift when red cells were suspended in Ringer solution, we compared the Cl⁻ concentrations in Ringer solutions before and after washing the cells. Within experimental errors of ±2 mEq/L, the cells did not gain any Cl⁻ (see Appendix D).

e. Discussion: The Significance of the Change in Liquid-Junction Potential

The liquid-junction potential at the tip of the microelectrode changes as the environment changes. For a microelectrode penetrating a red cell suspended in serum at 37°C, this change has been estimated to be maximally about ±0.3 mV (Lassen & Sten-Knudsen, 1968). That is to say, a possible error of ±0.3 mV occurs systematically in each measurement, due to possible change in the liquid-junction potential. The authors have assumed approximate values for the concentration of ions, and carried out the calculations using the limiting conductances, at infinite dilution, of the ions K⁺, Na⁺ and Cl⁻.
In our experiments, Ringer solution at 23°C was used. Concentrations of Na⁺ and Cl⁻ were somewhat different than in serum, and a re-evaluation of the junction potential change would be necessary. Moreover, the value of the conductance in the red cell cytoplasm is available in the literature and more realistic figures can be used. We have estimated the significance of the junction potential change, using the ionic conductances in a manner similar to that used by Cole & Moore (1960) in the study of the giant squid axon.

The total conductance of the cation and anion, in the red cell cytoplasm has been reported to be as high as 10.0 millimho-cm⁻¹ (Cook, 1952) and as low as 5.2 millimho-cm⁻¹ (Pauly & Schwan, 1966). The discrepancy represents variation by almost a factor of 2. The more recent data is not necessarily more accurate, and the range, rather than one of the two values should be considered. For our estimate of the problem, calculations were made using total conductances of 5.2, 7.5 and 10.0 millimho-cm⁻¹, representing the two reported values and one in between, approximately the mean of the two. It is most likely that the actual conductance in the red cell is within this range.

The red cell interior consists of KCl at a concentration of 0.1M, in addition to the high concentration of hemoglobin. A KCl solution at this concentration, and
at 25°C has a cation transference number of 0.4900. This means that the conductances of K⁺ and Cl⁻ in the solution are almost equal, and the ratio of cation to anion conductances is approximately equal to 1. The presence of negatively charged protein will modify the movement of ions in solution. The effect is that the cation movement will be impeded, and the cation-to-anion conductance ratio will be lowered. A reduction of the cation mobility, in the presence of protein, by as much as a factor of 4 has been reported (Overbeek, 1952, 1953). This represents a reduction of the cation-to-anion conductance ratio by the same factor. In the red cell where hemoglobin, with a net charge of about -4 is present at high concentration, one can expect a four or even six-fold reduction of the cation mobility. It is not likely that a factor of eight will be exceeded since it represents a rather drastic reduction. In our calculations, we have considered a 2, 4 and 8-fold reduction of the cation-to-anion conductance ratio.

Table 2 is a simplified version of a more detailed table shown in Appendix E. \((U_2 + V_2)\) is the total conductance of the cytoplasm, taken as 5.2, 7.5 and 10.0 millimho-cm\(^{-1}\) respectively in section I, II and III of the table. \(U_2/V_2\) is the cation-to-anion conductance ratio, taken as 0.48, 0.24 and 0.12 in lines A, B and C respectively for each value of the total conductance. These
Table 2. Change in Liquid-junction Potential  
As the Microelectrode Tip is Moved From  
Ringer Solution to the Red Cell Cytoplasm

<table>
<thead>
<tr>
<th></th>
<th>$U_2 + V_2$</th>
<th>$U_2/V_2$</th>
<th>$E_J$</th>
<th>$\Delta E_J$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>millimho-cm$^{-1}$</td>
<td>mV</td>
<td></td>
<td>mV</td>
</tr>
<tr>
<td>I</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>5.2</td>
<td>0.48</td>
<td>-2.78</td>
<td>-0.80</td>
</tr>
<tr>
<td>B</td>
<td>5.2</td>
<td>0.24</td>
<td>-2.30</td>
<td>-0.32</td>
</tr>
<tr>
<td>C</td>
<td>5.2</td>
<td>0.12</td>
<td>-2.00</td>
<td>-0.02</td>
</tr>
<tr>
<td>II</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>7.5</td>
<td>0.48</td>
<td>-2.35</td>
<td>-0.37</td>
</tr>
<tr>
<td>B</td>
<td>7.5</td>
<td>0.24</td>
<td>-1.72</td>
<td>+0.26</td>
</tr>
<tr>
<td>C</td>
<td>7.5</td>
<td>0.12</td>
<td>-1.34</td>
<td>+0.65</td>
</tr>
<tr>
<td>III</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>10.0</td>
<td>0.48</td>
<td>-1.91</td>
<td>+0.07</td>
</tr>
<tr>
<td>B</td>
<td>10.0</td>
<td>0.24</td>
<td>-1.09</td>
<td>+0.89</td>
</tr>
<tr>
<td>C</td>
<td>10.0</td>
<td>0.12</td>
<td>-0.64</td>
<td>+1.12</td>
</tr>
</tbody>
</table>

$U_2 + V_2$ is the total ionic conductance; $U_2/V_2$ is the cation-to-anion conductance ratio; $E_J$ is the liquid-junction potential between 3M-KCl and the cell cytoplasm; $\Delta E_J$ is the change in the liquid-junction potential moving the microelectrode from the Ringer solution to the cytoplasm. The liquid-junction potential between 3M-KCl and Ringer solution has been calculated to be -1.98 mV.
represent a 2, 4 and 8 fold reduction of 0.96, the cation-to-anion conductance ratio in 0.1M KCl at 25°C, in the absence of protein. $E_J$ is the liquid-junction potential between the 3M-KCl in the microelectrode and the cytoplasm. The liquid-junction potential between 3M-KCl in the microelectrode and the Ringer solution has been calculated to be -1.98 mV. $\Delta E_J$ is the change in liquid-junction potential when the microelectrode is moved from Ringer solution to the cytoplasm.

The maximum values of $\Delta E_J$ have been calculated to be -0.80 mV (line IA) and +1.12 mV (line IIIC). This means that if the lower value of the total conductance of 5.2 millimho-cm$^{-1}$ is correct, and there is only a 2 fold reduction of the conductance ratio, then our measurements would have been -0.80 mV high. At the other extreme, if the total conductance is 10.0 millimho-cm$^{-1}$, and if there is an 8 fold reduction of the conductance ratio in the cell, then our measurements would have been low by 1.12 mV. While the values in the table represent a wide range with extreme values, and any one may be the correct estimate, the extreme values are probably not very realistic. Because of the high hemoglobin concentration in red cells, we consider a 4 to 6 fold reduction of the conductance ratio to be reasonable (lines B and C in table). Rather than using one or the other reported value of the total conductance, we have considered the mean value to be a good estimate
(section II in table). Thus the values of $\Delta E_J$ calculated in lines IIB and IIC should be very good estimates of the change in the liquid-junction potential as the micro-electrode penetrates the membrane, i.e. our measurements probably had been low by about 0.5 mV.

Thus, although our results seemed to be low at first sight, when we consider the significances of a 20 per cent bound water in the cells, and a systematic error of about 0.5 mV due to the change in liquid-junction potential, our mean values are within the range of membrane potentials calculated by the Nernst equation.

3 RELATION BETWEEN MEMBRANE POTENTIAL AND PHYSIOLOGIC AGE OF HUMAN RED BLOOD CELLS

a. Results

Membrane potentials were measured from red cells in populations of 4 different ages separated according to density. Figures 22, 23, 24 and 25 show histograms of membrane potentials obtained from populations of young, young middle-age, old middle-age and old cells respectively. Each histogram includes all the results obtained for each age group.

Ninety-three young cells were studied from 5 samples taken from 3 subjects. These were cells taken from
Histogram of membrane potentials obtained from "YOUNG" cells, representing cells from the top 10 per cent of the packed cell column after separation according to density. These cells had the lowest densities in the samples, represented by cell profiles with no darkening.
YOUNG CELLS

\(-4.5 \pm 1.6\) (S.D.) mV

\(N = 93\)
FIGURE 23

Histogram of membrane potentials obtained from "YOUNG MIDDLE-AGE" cells, representing cells from the fourth 10 per cent of the packed cell column after separation according to density. These cells had medium light densities, and are represented by slight darkening of the cell profiles.
YOUNG MIDDLE AGE CELLS

\[-7.2 \pm 1.6 \text{(S.D.) mV}\]

\[N = 64\]
YOUNG MIDDLE AGE CELLS

\[-7.2 \pm 1.6 \text{(S.D.) mV}\]

\[N = 64\]
FIGURE 24

Histogram of membrane potentials obtained from "OLD MIDDLE-AGE" cells, representing cells from the seventh 10 per cent of the packed cell column after separation according to density. These cells had medium heavy densities, and are represented by darker shading.
OLD MIDDLE AGE CELLS
-5.7 ± 1.7 (S.D.) mV
N = 54
OLD MIDDLE AGE CELLS

-5.7 ± 1.7 (S.D.) mV

N = 54
FIGURE 25

Histogram of membrane potentials obtained from "OLD" cells, representing cells from the bottom 10 per cent of the packed cell column after separation according to density. These cells had the highest densities, and are represented by heavy shading.
OLD CELLS

-2.5 ± 1.1 (S.D.) mV

N = 71
OLD CELLS
-2.5 ± 1.1 (S.D.) mV
N = 71
the top 10 per cent of packed cell columns, and had the lowest densities within the whole cell population. The membrane potentials obtained ranged from -1.0 mV to -8.8 mV, with a mean value of -4.5 ± 1.6 (S.D.) mV.

Sixty-four young middle-age cells were studied from 5 samples taken from 2 subjects. These cells were from the fourth 10 per cent of the packed cell columns. The membrane potentials ranged from -2.8 mV to -11.5 mV, with a mean value of -7.2 ± 1.6 (S.D.) mV.

Fifty-four old middle-age cells were studied from 5 samples taken from 2 subjects (same samples as those studied in the young-middle-age populations). These cells were from the seventh 10 per cent of packed cell columns. The membrane potentials ranged from -2.3 mV to -9.5 mV, with a mean value of -5.7 ± 1.7 (S.D.) mV.

Seventy-one old cells were studied from 6 samples taken from 4 subjects. These cells were taken from the bottom 10 per cent of packed cell columns and had the highest densities within the whole population. The membrane potentials ranged from -1.0 mV to -5.2 mV, with a mean of -2.5 ± 1.1 (S.D.) mV.

b. Discussion

With the exception of that of the old cells, the histograms resemble normal distributions of membrane potentials
with wide ranges of values and large standard deviations. The histogram of the old cells is skewed to the left, towards lower values, and the standard deviation is smaller. One would expect that, because of the enrichment of cells of particular ages in different parts of the packed cell column, the standard deviations in individual age groups would be significantly lower than that in mixed age studies. This is not the case as our results indicate. While the standard deviations remain approximately the same, the mean values vary with age of the cell. The amount of variation in the mean values with age is larger than the variation expected among different samples and different subjects.

Figures 26 and 27 show plots of the mean membrane potential against the relative mean cell age according to density. In Figure 26, the individual sample means are shown. The number of cells studied in each sample ranged from 7 to 35. The error bars show the maximum and minimum ±S.E.M. (standard error of mean) of the points. There is some variation of the mean membrane potential from sample to sample in each age group. The variation has been caused partly by the variations which occur from sample to sample and from subject to subject, and partly by the fact that some samples involved only small numbers of cells. There is considerably more variation in the young and old cells than in middle age cells. This could have been caused by
FIGURE 26

Relation between mean membrane potential and physiological age of human red blood cells. The points shown are mean values of different blood samples studied. The error bars show the maximum and minimum values of $\pm$S.E.M. among the samples.

FIGURE 27

Relation between mean membrane potential and physiological age of human red blood cells. Error bars show $\pm$S.E.M., means calculated from all measurements obtained for each age group. The four different ages only represent the relative cell ages according to increasing density, and the populations studied only represent populations "enriched" with cells of a particular age group.
inconsistency of the cell separation procedure. In figure 27, the overall means for individual age groups are shown. The error bars represent ±S.E.M.

With the exception of the young cells, the membrane potential decreases with age, suggesting a decrease in intracellular chloride ratio with age. Despite the scatter in the sample means, the general shape of the curve is reproducible. It has been reported that intracellular chloride concentration decreases progressively with age (Keital et al., 1955; Borun, 1963). The chloride concentration in cells from the top, middle and bottom fractions of the centrifuged cell column were found to be 80.7, 80.2 and 79.2 mEq/L cell H₂O respectively (Borun, 1963). The change in concentration of 1.5 mEq/L cell H₂O over the whole life-span of the cell represents only about 1.5 per cent increase in the chloride ratio, or about 0.2 mV increase in the membrane potential. It is doubtful whether such a small change could be detected by microelectrode measurements in single cells. Although our study involves a significantly large number of measurements, the confidence in the mean values is far from those obtained in biochemical analysis where individual samples involve billions of red cells.
The breakdown of the metabolic process has been associated with in vivo aging of the red cell. In addition to a slight decrease in Cl\(^{-}\) concentration, the cell gains Na\(^{+}\) and loses K\(^{+}\) (Bernstein, 1959). This lowers the transmembrane concentration gradients of both Na\(^{+}\) and K\(^{+}\), but raises that of Cl\(^{-}\). In vitro studies have shown that metabolism of the red cell also declines with storage in the cold, and concentration gradients of Na\(^{+}\), K\(^{+}\) and Cl\(^{-}\) across the membrane all decrease, with those of Na\(^{+}\) and Cl\(^{-}\) approaching unity at minimum metabolism (Harris & Maizels, 1952). While in vivo aging and prolonged storage in vitro have similar effects on the Na\(^{+}\) and K\(^{+}\) concentration gradients, as a result of changes in membrane permeability and function, they seem to have opposite effect on the Cl\(^{-}\).

We can only speculate about the reason for the peculiar relation between membrane potential and cell age indicated by our results. Intuitively, one would expect a reduction of the Cl\(^{-}\) gradient with age. If metabolism stops in the cell, the situation will approach a Donnan equilibrium. Although hemoglobin is present in the cell at a very high concentration, the equivalent concentration is
very low because of the high molecular weight of the protein, and therefore the Cl⁻ ratio should approach unity, i.e. the intracellular Cl⁻ concentration should increase. This line of thinking is consistent with results of the in vitro experiments of Harris & Maizels (1952). The effect of metabolic decline due to storage probably does not apply in our experiments since our results from washed and unwashed cells showed no significant difference in the membrane potentials within the whole population although the mean value in washed cells turned out to be 0.4 mV lower. Nevertheless, if the chloride ratio is related to the metabolism of the cell, then our results are consistent with what might be expected.

It is not clear why the mean membrane potential of the young cells turned out to be lower than that of the young middle age cells. One explanation may be because of reticulocyte contamination. In normal blood, reticulocytes are present at as high as 1.5 per cent. Upon centrifugation, if all the reticulocytes appear in the top 10 per cent of the cell column, because they have lower densities than mature red cells, then the reticulocyte count can be as high as 15 per cent. Harris & Maizels (1952) have pointed out that the fall in Cl⁻ ratio during storage is associated with a rise in pH, rather than directly with the state of metabolism, since it was found that approximately, \([\text{Cl}^-]_o/\text{[Cl}^-]_i = [\text{H}^+]_i/[\text{H}^+]_o\).

Calvey (1970) has shown that reticulocytes have higher pH than
normal cell populations, which mean that reticulocytes would have a lower Cl\(^-\) ratio than the mean of the population.

If we had studied the young cells and the reticulocytes indiscriminately and if reticulocytes have lower membrane potentials than matured young cells, then the mean membrane potential as measured would be lower than the true mean for young matured cells alone. Supposing that 15 of the 93 cells we studied in the young population were actually reticulocytes and supposing these 15 had the lowest recorded potentials, then a calculation shows that if we exclude these 15 cells, the mean for the young cells alone will be about -5 mV. This is still lower than expected.

One impression we had during our studies of young cell populations was that measurements were easier to obtain from young cell populations than from mixed age samples or from older cells. If this relative ease is due to our actually studying reticulocytes, then even more than 15 per cent of our measurements could have been from reticulocytes. For example, if every reticulocyte studied yielded a measurement but only one in five attempts on matured young cells did so, then about 50 per cent of our measurement would have been from reticulocytes. Supposing this happened and supposing the reticulocytes yielded lower potentials, the young cells could have a mean membrane potential of higher than -6 mV.

Another possibility to be considered is the liquid-junction problem. Since older cells have less water, the
concentration of hemoglobin is higher than in younger cells. The cation mobility inside the cell could be further impeded, resulting in a larger positive change in the junction potential, and a larger under-estimate of the membrane potential. Fortunately, even if the cation mobility is further reduced by a factor of 2, the amount of under-estimate in the membrane potential can only be another fraction of a millivolt.

The tendency of old cells from the bottom of the centrifuged cell columns to crenate could have contributed to lower membrane potentials. Our study of crenated cells (see following section) in normal samples had resulted in low positive membrane potentials. The old cells studied were not crenated however, but it might be possible that the lower recorded values were associated with the tendency to crenate.

All of the possible explanations suggested above are purely speculations and must be taken as no more than such. It is not possible to determine if any of them does apply until the possibilities have been explored experimentally. Our age study of the membrane potential suggests that the "effective" chloride concentration inside the cell increases with the age of the cell.
(4) MEMBRANE POTENTIALS IN CRENATED CELLS

a. Results

Forty-four measurements were made in crenated cells from 2 samples in different subjects. In contrast to negative potentials obtained for normal cells, all measurements from crenated cells gave positive values, ranging from +0.2 mV to +1.0 mV. The overall mean value was +0.5 ± 0.2 (S.D.) mV. The two individual samples had mean values differing by 0.1 mV.

b. Discussion

As described earlier in the methods section, the crenations in the cells studied were crenations due to contact with the cover-slip. The potentials obtained were very stable, and some cells had withstood repeated penetrations by the microelectrode before hemolysis occurred. This was probably because the membranes in the crenated cells were slack and without tension, and sealing around the microelectrode was more effective than in normal biconcave cells. The slight positive potentials recorded meant that within measurement errors, the membrane potential had decreased to zero upon crenation. This could result from one or more of three possibilities. Contrary to Ponder's (1958) observation that such crenations were unaccompanied
by volume change, the cells could have lost water. A volume decrease of up to 20 per cent, the amount of water loss required for the chloride ratio to approach unity, might not have been detected in Ponder's qualitative observations. Secondly, the crenations might have resulted in the cells gaining chloride with or without loss of water, causing the Cl\textsuperscript{-} ratio to approach unity. An increase in cellular pH could have caused an increase in intracellular Cl\textsuperscript{-} concentration and therefore have lowered the membrane potential. The crenations are known to be associated with charge phenomena as well as a drastic change in pH (Ponder, 1958). Rand et al. (1965) have also shown that red cells crenate reversibly with change in pH induced by an electric field. The crenations took place in cells near the anode, where the pH was high. If the high pH in the medium caused an increase in cell pH, then the intracellular chloride could increase. Thirdly, if the crenation caused by a violent pH change resulted in an increase in water binding within the cell, the effective intracellular Cl\textsuperscript{-} concentration would rise. An increase from 20 to 30 per cent bound water would account for the observed change in the membrane potential.

Finally, the magnitude of the potentials obtained were very close to the calculated change in liquid-junction potential as the microelectrode entered the cell. Therefore,
it was possible that crenations had caused the membrane potential to approach zero, and the measured deflections actually were measures of the liquid-junction potential change as the microelectrode entered the cell. The observations that the deflections were very constant, and that repeated measurements on the same cell gave the same value, supports this view.

One final observation was that the several normal shaped cells in the same samples which were studied gave negative deflections of up to -2 mV.

(5) MEASUREMENTS FROM RED CELL AGGREGATES

a. Results

Figure 28 shows typical recordings obtained from cell aggregates. Forty-two measurements were made. The cells were placed in plasma. An initial sharp positive deflection occurred as the microelectrode was advanced through the surface into the aggregate and left undisturbed. The initial deflections gave a mean value of \(+5.0 \pm 2.4\) (S.D.) mV. The deflection decayed quickly to a lower positive value which remained constant until the microelectrode was retracted and the deflection returned to the zero-line. The mean of the constant potential following the rapid decay was \(+2.1 \pm 0.8\) (S.D.) mV. The mean of the
FIGURE 28

Typical recordings obtained from studies of red cell aggregates. All potentials measured were positive. Right lower trace shows recording obtained when the microelectrode was continually advanced into the aggregate. Other traces show single penetrations.
magnitude of decay, i.e. the difference between the initial maximum and the constant value which followed was $-3.3 \pm 2.0$ (S.D.) mV.

Two observations were of particular interest. Firstly, the deflection never returned to zero as long as the microelectrode tip was inside the aggregate. Secondly, if the microelectrode tip was further advanced, another sharp deflection was observed, this again rapidly decayed to a lower constant value which might or might not be equal to the original constant value. This procedure could be carried out repeatedly.

b. Discussion

Because of optical limitations, it was not possible to see the exact location of the tip of the microelectrode inside the aggregate. Thus, whether the microelectrode tip was inside a cell or in the intercellular space at any time was not known. While studies of several single cells in the same sample produced negative potentials of several millivolts magnitude, all the measurements in the aggregates produced positive deflections.

It is not known whether there is really a reversal of the membrane potential in aggregated cells. Lassen & Sten-Knudsen (1968) have also reported positive potentials from cell aggregates. One thing was quite clear from our
measurements. At one time or another, the microelectrode must had been in the intercellular space within the cell aggregate. Since the deflection was always positive as long as the microelectrode tip was inside the aggregate, the intercellular medium inside the aggregate must be positive with respect to the ground electrode, or the plasma.

One approach to the problem involves the possible effect of fixed charges, on the surface of the aggregate and in the intercellular space. Assuming that the charge effect caused the initial sharp positive deflection of +5.0 mV, then the subsequent change of -3.3 mV, with respect to the maximum, would represent the membrane potential difference between the cell and the intercellular space. Thus, although the cell interior was still positive with respect to the plasma medium outside the aggregate, it was -3.3 mV negative with respect to the intercellular space within the aggregate, i.e. the environment immediate to the cell. If this was true, then two observations were worth noting. Firstly, the potential measurement showed rather slow response. This could have been caused by the charge effect having increased the input capacitance, and neutralization by the negative capacitance was no longer adequate, resulting in much slower response and longer rise-time in the amplifier. Secondly, the membrane potentials, after the slow response, were very stable, as compared to those obtained from single
cells. This was not entirely surprising since the cells were probably quite tightly packed in the aggregates. If the intercellular space was only several percent of the aggregate volume, then even if a cell hemolysed upon penetration by an electrode, there would not be extensive leakage of the cell content. In effect, the neighbouring cells served as a closed boundary and the microelectrode saw no change upon hemolysis of the cell. Thus, the deflection could remain quite constant. The increase in noise level when the microelectrode was inside the aggregate suggested very high resistance between the electrode tip and ground, this is consistent with the speculation that leakage would be minimal.

The value of $-3.3$ mV agreed quite well with the expected membrane potential, if we consider that the medium used was plasma, having lower Cl$^-$ than our Ringer solution, therefore the Cl$^-$ ratio would be comparatively lower and the expected membrane potential should have been about $-3$ mV.

Although we favour the charge phenomenon approach to the problem, the possibility that there actually had been a reversal of membrane potential must not be disregarded. If the membrane potential had become positive, i.e. the intracellular Cl$^-$ concentration had become higher than in the plasma, then the initial spike of $+5.0 \pm 2.0$ (S.D.) mV would be the membrane potential, and the subsequent lower
stable value of $+2.1 \pm 0.8$ (S.D.) mV would represent the potential in the intracellular space with respect to the plasma medium. The reversal of the membrane potential in aggregated cells could be a result of drastic changes in the permeabilities when cells aggregate. There is no evidence in the literature which directly supports this view, although modification of membrane permeabilities at tight junctions between cells has been reported (Loewenstein, 1966) and an increase in $K^+$ efflux in human red cells has been associated with reversing the sign of the membrane potential (Cotterrell & Whittam, 1970).

While measurements in single cells were extremely difficult, those in aggregates were easy to obtain. The study of degree of cell aggregation is a diagnostic tool in clinical medicine. Since the "charge effect" transients and the steady potentials that follow are related to aggregation, perhaps, with some refinement, the method of microelectrode study could be developed to become a routine procedure with some clinical application.

(6) MEMBRANE POTENTIALS IN WHITE CELLS

a. Results

Membrane potentials were obtained from 18 white cells. The values ranged from $-2.8$ mV to $-7.8$ mV with a
mean value of $-4.5 \pm 1.5$ (S.D.) mV. The measurements appeared very similar to those obtained from normal red cells but the deflections were more stable and almost every attempt gave a potential reading.

b. Discussion

The measurements of membrane potentials from white cells were obtained from white cells observed in various samples during the course of our study. The individual white cells were not typed according to classification, nor were we able to say whether the microelectrode was in any specific part of the cell. The study was made only because the cells were conveniently present in the samples and some measurements would be interesting for comparison with the red cells. Since our measurements, Beckmann et al. (1969) has reported a more detailed study. Our results are in good agreement. The authors have reported a range of $-1$ mV to $-10$ mV, with a mean of $-5$ mV for 289 measurements.

(7) MEASUREMENT FROM SEMIPERMEABLE NYLON MICROCAPSULES CONTAINING HEMOGLOBIN SOLUTION

a. Results

Thirty-four microcapsules containing hemoglobin
solution were studied by microelectrode punctures. Figure 29 shows two typical examples of recordings obtained from microcapsules. A very sharp negative spike was recorded as the microelectrode punctured the nylon membrane. This was followed by a slowly increasing negative deflection which reached a maximum value usually in about 5 to 10 seconds and then decayed slowly to a smaller constant negative deflection. The deflection sharply returned to zero when the microelectrode was retracted. The maximum transient deflection had a mean value of $-1.7 \pm 0.7$ (S.D.) mV and the final stable deflection had a mean value of $-0.5 \pm 0.1$ (S.D.) mV.

b. Discussion

Nothing is known about the initial transient deflection although it is suspected that it might be a result of some charge phenomenon. Unfortunately, the diameter of individual microcapsules were not measured, therefore the magnitude of the transient potential cannot be correlated with the surface area and volume. Some microcapsules did not rupture completely upon retraction of the microelectrode. The microelectrode was then advanced immediately into the microcapsule a second time. The transient potential did not occur at the second penetration, but the constant potential that followed was reproducible.
FIGURE 29

Typical recordings obtained from studies of semipermeable nylon microcapsules containing hemoglobin solution.
The smaller, stable deflection of -0.5 mV was exactly what was calculated for the Donnan potential across the nylon membrane. Thus, there was no measurable change in the liquid-junction potential as the microelectrode penetrated the membrane. These experiments served as a test for our measuring system. Since the hemoglobin concentration in the microcapsules was only about 20 per cent of that in the red cell, the results do not imply that the liquid-junction potential problem could be neglected in the red cell experiments. On the other hand, if these results had not agreed with the calculated values, then the problem in the red cell experiments would quite possibly have been serious.
X. SUMMARY AND CONCLUSIONS

The primary aim of this thesis was to develop the microelectrode techniques to a sufficient degree so that the electrical potential difference across the red cell membrane could be directly measured. It has generally been assumed that the potential across the red cell membrane is a "chloride" potential, i.e. that chloride is passively distributed across the membrane, and that the potential can be calculated by the Nernst equation considering the transmembrane concentration ratio of the chloride ions alone. This assumption has been based on the observation that the ionic permeability of the red cell membrane to chloride is extremely high compared with those to sodium and potassium. The validity of the assumption is best evaluated by direct measurement and by comparing the measured value with the calculated value.

Several difficulties were expected in direct measurements. Firstly, the red cell is small, with a diameter of only 8μ and a maximum thickness of little more than 2μ. This increases the effect of damage to the membrane resulting in leakage during measurements. Secondly, the
membrane potential has been calculated, from the trans-
membrane chloride ratio, to be only about -10 mV. In
measurements of such small potentials, the change in the
liquid-junction potential at the microelectrode tip as it
goes through the membrane is not negligible. Since the
magnitude of this change cannot be measured experimentally,
it must be estimated by considering extreme conditions which
can possibly exist in the intact cell.

The secondary aim of this thesis was to apply the
developed techniques to study the age dependence of the mem-
brane potential. Various investigators have shown that the
metabolism of the red cell decreases with in vivo aging
during its life span of 120 days. While some investigators
have reported that the intracellular chloride concentration
decreases slightly with age, others have shown by in vitro
experiments that it increases and approaches the extra-
cellular concentration as the metabolic pump slows down and
approaches a minimum during storage. Biochemical techniques
provide a measure of the intracellular chloride concentra-
tion by considering the total chloride and total water in
the cell. Because of the 20 per cent "bound water" in the
red cell, the "effective" chloride concentration in the in-
tact cell is higher than the "measured" value by a factor of
1.25. The microelectrode sees the "effective" concen-
tration as it enters the cell, therefore the recorded membrane
potentials must be compared to that calculated from the "effective" rather than the "measured" values.

This investigation has led to sufficient development of technique, mainly the use of a microelectrode rapid-advance mechanism, to allow accurate measurement of the membrane potential. By excluding the 20 per cent bound water in the cell, we have shown that the measured value compares very well with the predicted value, thus strongly supporting the theory that the potential across the red cell membrane is a chloride potential, i.e. that chloride is passively distributed across the membrane.

The change in the liquid-junction potential, as the microelectrode entered the cell interior, has been estimated. The change has probably caused our measurements to be low, maximally by about 0.5 mV.

The age dependence of the membrane potential has been studied. The potential increases with age during the first half of the cell's life span, reaching a maximum at about early middle age, and decreases with further aging. The peaking at early middle age has been discussed with reference to similar peaking reported in the literature for studies of immune lysis of red cells and percentage glucose-to-lactate conversion, both of which are metabolic dependent. The membrane potential does appear to be directly related to the metabolism of the cell.
Membrane potentials have also been measured in red cells which were crenated by contact with glass coverslips, in red cell aggregates, in white cells, and in semipermeable nylon microcapsules containing hemoglobin solution. The conclusions drawn from this investigation are summarized below.

CONCLUSIONS

The electrical potential difference across the human red cell membrane has been successfully measured using ultra-fine glass microelectrodes filled with 3M-KCl. A rapid-advance mechanism was designed and used to drive the microelectrodes into the cells.

1. The recorded membrane potentials ranged from -1 mV to -13 mV, the cell interior being negative with respect to the extracellular medium. The mean value for a cell population was found to be $-5.6 \pm 1.6$ (S.D.) mV. The wide range of potentials within a population suggested that there might be an age or metabolic dependence of the potential.

2. The mean membrane potential varied from subject to subject and from sample to sample. The variation in each case was as much as 1 mV. Some variation of the mean value could be a result of relatively small numbers
of cells studied in individual samples.

3. The mean value fell within the range predicted by the
Nernst equation using the reported range of intracellular
chloride concentrations in the calculations, and taking
into account that 20 per cent of the cell water in the
intact cell was not available to chloride ions.

4. Our results showed that the effective chloride con-
centration is higher than the biochemically measured
value and seems to support the concept of bound water,
a subject of much dispute among investigators. The
observation, however, is consistent with those from
microelectrode studies on muscles, where bound water
has also been reported. The good agreement between the
predicted and measured potentials supports the theory
that the potential across the red cell membrane is a
chloride potential, that chloride is passively distrib-
buted across the membrane.

5. The change in the liquid-junction potential as the
microelectrode penetrates the membrane has been
estimated by assigning possible extreme values to the
Henderson equation, and found to be positive and
maximally about 0.5 mV. Our measurements, therefore,
could be low, by at most 0.5 mV.

6. The age dependence of the membrane potential has been
studied. Cells of different mean ages were obtained by
centrifugation. The study of separated and re-mixed
samples showed that the process of washing and centri-
fugation had not altered the mean membrane potential of the cell population.

7. With the exception of the very young cells, the membrane potential decreases with age. The lower than expected mean value obtained for the young cells was probably due to reticulocyte contamination.

8. Red cells which crenated upon contact with glass coverslips had low positive potentials. The measured value of about +0.5 mV was very close to the expected change in liquid-junction potential. That the membrane potential approached zero upon crenation implied either or both an increase in intracellular chloride concentration with an increase in intracellular pH, or an increase in percentage water binding, from 20 to 30 per cent.

9. Studies of red cell aggregates in serum showed that the potentials inside the cells and in the intracellular spaces within the aggregates were positive with respect to the suspending medium. The cell interior was probably still negative with respect to its immediate environment. The reversal in the sign of the potential has been discussed in terms of permeability changes and of surface charge phenomenon.
10. Membrane potentials in white cells were studied. The values ranged from about -2 mV to -8 mV, with a mean of -4.5 mV. The results were consistent with those recently reported by other investigators.

11. Studies in semipermeable nylon microcapsules containing hemoglobin solutions showed that after a large slow initial transient, which was probably a charge phenomenon, the potential decreased to a constant, lower value which corresponded to the calculated Donnan potential. This implied that the change in liquid-junction potential, as the microelectrode entered an environment of hemoglobin solution, was negligible.
REFERENCES


XV. Hydration of macrosized crystals of human
hemoglobin and osmotic concentration in red cells.
J. Biol. Chem. 185:231-245.

elasticity of red cells and small blood vessels.

sphering of red blood cells. Biophys. J. 8:
175-198.

erythrocytes and hemoglobin solutions. J. Gen.
Physiol. 50:2547-2564.

of hemoglobin solutions in red cells. J. Gen.
Physiol. 52:825-853.

Donan relation in the giant barnacle striated

42. Glynn, I. M. (1957a). The ionic permeability of the

43. Glynn, I. M. (1957b). The action of cardiac glycosides
on Na⁺ and K⁺ movements in human red cells.

44. Goldman, D. E. (1943). Potential, impedance and rectifi-


125. Westerman, M. P., Pierce, L. E. & Jenson, W. N. 


    Lea & Febiger, Philadelphia, U. S. A.

    Blood. 33:159-163.
APPENDIX A

PREPARATION OF MODIFIED ISOTONIC BUFFERED RINGER SOLUTION
MODIFIED ISOTONIC BUFFERED RINGER SOLUTION

SOLUTION A: Modified isotonic Ringer solution.

B: Isotonic HCl solution.

C: Isotonic Tris-Hydroxymethyl aminomethane solution.

Table 3 Materials used for preparation of Ringer solution and buffer solution

<table>
<thead>
<tr>
<th>MATERIAL</th>
<th>AMOUNT (grams)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td></td>
</tr>
<tr>
<td>NaCl</td>
<td>8.32</td>
</tr>
<tr>
<td>KCl</td>
<td>0.42</td>
</tr>
<tr>
<td>CaCl₂</td>
<td>0.24</td>
</tr>
<tr>
<td>NaHCO₃</td>
<td>0.20</td>
</tr>
<tr>
<td>C₆H₁₂O₆ (dextrose)</td>
<td>1.00</td>
</tr>
<tr>
<td>B</td>
<td></td>
</tr>
<tr>
<td>HCl</td>
<td>5.66</td>
</tr>
<tr>
<td>C</td>
<td></td>
</tr>
<tr>
<td>C₄H₁₁NO₃</td>
<td>37.60</td>
</tr>
</tbody>
</table>
Each of A, B and C was added to distilled water to make up 1 litre of solution at 310 mOsm. A Fiske osmometer was used.

MIXING PROCEDURE:

1. The buffer system was prepared by mixing 120ml of solution B with 80ml of solution C. The mixture was adjusted to pH 7.40 (±0.02) using a Beckman pH meter.

2. The 200ml mixture were added to 800ml of the prepared Ringer, solution A.

3. The resultant solution was 1 litre of modified isotonic (310±5 mOsm) buffered (pH 7.40±0.02) Ringer solution. The final concentrations of the three major ions were calculated to be:

   \[
   \begin{align*}
   \text{Na}^+ & \quad 110 \text{ mEq/L} \\
   \text{K}^+ & \quad 4.5 \text{ mEq/L} \\
   \text{Cl}^- & \quad 140 \text{ mEq/L}
   \end{align*}
   \]

4. The solution was filtered through a 0.22μ millipore filter before used.
Table 4  Final ionic concentrations in the modified isotonic buffered Ringer solution

<table>
<thead>
<tr>
<th>ION</th>
<th>mEq/L</th>
</tr>
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<tr>
<td>$K^+$</td>
<td>4.5</td>
</tr>
<tr>
<td>$Na^+$</td>
<td>110</td>
</tr>
<tr>
<td>$Cl^-$</td>
<td>140</td>
</tr>
<tr>
<td>$Ca^{++}$</td>
<td>3.5</td>
</tr>
<tr>
<td>$HNO_3^-$</td>
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APPENDIX B

TABLES OF RESULTS
TABLE 5. NON-DEPENDENCE OF MEAN MEMBRANE POTENTIAL ON MICROELECTRODE RESISTANCE

<table>
<thead>
<tr>
<th>Sample</th>
<th>$R_e$</th>
<th>No. Cells</th>
<th>Mean ± (S.D.) mV</th>
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</thead>
<tbody>
<tr>
<td>Mix-Nw</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AJ-2</td>
<td>16</td>
<td>6</td>
<td>-6.3 ± 2.0</td>
</tr>
<tr>
<td>AJ-2</td>
<td>25</td>
<td>9</td>
<td>-5.2 ± 1.1</td>
</tr>
<tr>
<td>AJ-3</td>
<td>12</td>
<td>7</td>
<td>-4.9 ± 1.1</td>
</tr>
<tr>
<td>JS-1</td>
<td>25</td>
<td>10</td>
<td>-6.7 ± 1.5</td>
</tr>
<tr>
<td>AB-1</td>
<td>22</td>
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<td>-5.1 ± 1.0</td>
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<tr>
<td>PF-1</td>
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<td>-6.7 ± 2.3</td>
</tr>
<tr>
<td>CW-1</td>
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<td>13</td>
<td>-5.9 ± 1.1</td>
</tr>
<tr>
<td>CW-2</td>
<td>18</td>
<td>9</td>
<td>-5.2 ± 1.4</td>
</tr>
</tbody>
</table>

Mix-Nw - Mixed age cell samples, not washed.
Mix-W - Mixed age cell samples, washed, separated and re-mixed.
$R_e$ - Microelectrode resistance in MΩ.
TABLE 6. MEAN MEMBRANE POTENTIALS: SAMPLE MEANS, SUBJECT MEANS AND OVERALL MEANS

<table>
<thead>
<tr>
<th>SAMPLE</th>
<th>SAMPLE MEAN</th>
<th>SUBJECT MEAN</th>
<th>OVERALL MEAN</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mixed</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AJ-2</td>
<td>-5.6±1.6(15)</td>
<td>-5.4±1.5(22)</td>
<td></td>
</tr>
<tr>
<td>Not</td>
<td></td>
<td></td>
<td>-5.6±1.6(46)</td>
</tr>
<tr>
<td>AJ-3</td>
<td>-4.9±1.1(7)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Washed</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>JS-1</td>
<td>-6.7±1.5(10)</td>
<td>-6.7±1.5(10)</td>
<td></td>
</tr>
<tr>
<td>AB-1</td>
<td>-5.1±1.0(14)</td>
<td>-5.1±1.0(14)</td>
<td></td>
</tr>
<tr>
<td>Washed</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PF-1</td>
<td>-4.1±1.7(15)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mixed</td>
<td></td>
<td></td>
<td>-5.2±1.8(72)</td>
</tr>
<tr>
<td>PF-2</td>
<td>-4.6±1.3(13)</td>
<td>-4.4±1.5(37)</td>
<td></td>
</tr>
<tr>
<td>PF-3</td>
<td>-4.7±1.1(9)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Washed</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CW-1</td>
<td>-6.3±1.8(26)</td>
<td>-6.0±1.8(35)</td>
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<tr>
<td>CW-2</td>
<td>-5.2±1.4(9)</td>
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<td>Young Cells</td>
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<td></td>
<td></td>
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<tr>
<td>JS-2</td>
<td>-4.4±1.3(20)</td>
<td>-4.2±1.8(55)</td>
<td></td>
</tr>
<tr>
<td>JS-3</td>
<td>-4.1±1.7(35)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AB-2</td>
<td>-5.3±1.6(9)</td>
<td>-5.3±1.6(9)</td>
<td>-4.5±1.6(93)</td>
</tr>
<tr>
<td>Young Middle Age</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PF-9</td>
<td>-3.9±1.3(17)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PF-10</td>
<td>-5.7±1.4(12)</td>
<td>-4.6±1.6(29)</td>
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</tr>
<tr>
<td>Young Middle Age</td>
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<td></td>
</tr>
<tr>
<td>PF-4</td>
<td>-7.4±1.7(16)</td>
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<tr>
<td>PF-5</td>
<td>-7.2±2.2(12)</td>
<td>-7.1±1.9(57)</td>
<td>-7.2±1.6(64)</td>
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<tr>
<td>PF-6</td>
<td>-6.6±1.6(10)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PF-7</td>
<td>-7.2±1.9(19)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CW-3</td>
<td>-7.4±1.6(7)</td>
<td>-7.4±1.6(7)</td>
<td></td>
</tr>
</tbody>
</table>
### TABLE 6. (CONTINUED)

<table>
<thead>
<tr>
<th>SAMPLE</th>
<th>SAMPLE MEAN</th>
<th>SUBJECT MEAN</th>
<th>OVERALL MEAN</th>
</tr>
</thead>
<tbody>
<tr>
<td>Old PF-4</td>
<td>$-5.8\pm1.4$ (8)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Old PF-5</td>
<td>$-5.9\pm1.5$ (17)</td>
<td>$-5.7\pm1.6$ (46)</td>
<td></td>
</tr>
<tr>
<td>Middle PF-6</td>
<td>$-5.5\pm1.2$ (7)</td>
<td>$-5.7\pm1.7$ (46)</td>
<td>$-5.7\pm1.7$ (54)</td>
</tr>
<tr>
<td>Age PF-7</td>
<td>$-5.6\pm1.9$ (14)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CW-3</td>
<td>$-5.4\pm2.1$ (8)</td>
<td>$-5.4\pm2.1$ (8)</td>
<td></td>
</tr>
<tr>
<td>Old JS-2</td>
<td>$-1.7\pm0.5$ (23)</td>
<td>$-2.0\pm0.7$ (33)</td>
<td></td>
</tr>
<tr>
<td>Old JS-3</td>
<td>$-2.6\pm0.7$ (10)</td>
<td>$-2.2\pm0.6$ (7)</td>
<td>$-2.5\pm1.1$ (71)</td>
</tr>
<tr>
<td>Old AB-2</td>
<td>$-2.2\pm0.6$ (7)</td>
<td>$-2.2\pm0.6$ (7)</td>
<td>$-2.4\pm0.9$ (21)</td>
</tr>
<tr>
<td>Cells CW-3</td>
<td>$-4.4\pm0.6$ (10)</td>
<td>$-4.4\pm0.6$ (10)</td>
<td></td>
</tr>
<tr>
<td>Cells PF-9</td>
<td>$-2.4\pm1.1$ (14)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cells PF-10</td>
<td>$-2.3\pm0.5$ (7)</td>
<td>$-2.4\pm0.9$ (21)</td>
<td></td>
</tr>
<tr>
<td>Crenated AJ-4</td>
<td>$+0.5\pm0.2$ (34)</td>
<td>$+0.5\pm0.2$ (34)</td>
<td>$-0.5\pm0.2$ (44)</td>
</tr>
<tr>
<td>Cells KS-1</td>
<td>$+0.6\pm0.2$ (10)</td>
<td>$+0.6\pm0.2$ (10)</td>
<td></td>
</tr>
<tr>
<td>Init-Max</td>
<td>$+5.0\pm2.4$ (42)</td>
<td>$+5.0\pm2.4$ (42)</td>
<td>$+5.0\pm2.4$ (42)</td>
</tr>
<tr>
<td>Aggr. Final-Const</td>
<td>$+2.1\pm0.8$ (42)</td>
<td>$+2.1\pm0.8$ (42)</td>
<td>$+2.1\pm0.8$ (42)</td>
</tr>
<tr>
<td>Difference</td>
<td>$-3.3\pm2.0$ (37)</td>
<td>$-3.3\pm2.0$ (37)</td>
<td>$-3.3\pm2.0$ (37)</td>
</tr>
<tr>
<td>White Cells</td>
<td></td>
<td></td>
<td>$-4.5\pm1.5$ (12)</td>
</tr>
</tbody>
</table>

Numbers in brackets indicate number of cells in the population.
APPENDIX C

CALCULATION OF DONNAN POTENTIAL
ACROSS THE SEMI-PERMEABLE NYLON
MICROCAPSULE MEMBRANE
CALCULATION OF DONNAN POTENTIAL IN MICROCAPSULES

Hemoglobin concentration in red cells = 340 gm/L
Hemoglobin concentration in microcapsules = 68 gm/L

Molecular Weight of Hb = 68,000
Charge of Hb molecule = -4 at pH 7.4

\[ .68 \text{ gm/L} = 68 \times \frac{4}{68,000} \]
\[ = 0.0040 \text{ Eq/L} \]

Assuming Ringer solution is 0.9% NaCl, it will contain
0.1538 Eq/L Na⁺
0.1538 Eq/L Cl⁻

Therefore,
\[ [\text{Hb}^-]_i = 0.0040 \text{ Eq/L} \]
\[ [\text{Cl}^-]_o = 0.1538 \text{ Eq/L} \]
\[ [\text{Na}^+]_o = 0.1538 \text{ Eq/L} \]
To calculate $[\text{Na}^+]_i$ and $[\text{Cl}^-]_i$

Donnan conditions for equilibrium:

(1) $[\text{Hb}^-]_i + [\text{Cl}^-]_i = [\text{Na}^+]_i$

$\therefore [\text{Na}^+]_i = [\text{Cl}^-]_i + 0.004$

(2) $[\text{Cl}^-]_i [\text{Na}^+]_i = [\text{Cl}^-]_o [\text{Na}^+]_o$

$\therefore [\text{Cl}^-]_i [\text{Na}^-]_i = 0.02365$

$\therefore [\text{Cl}^-]_i^2 + 0.004[\text{Cl}^-]_i - 0.02365 = 0$

solving quadratic equation

$[\text{Cl}^-]_i = 0.1518 \text{ Eq/L}$

$\therefore [\text{Na}^+]_i = 0.1558 \text{ Eq/L}$

$
\frac{[\text{Cl}^-]_o}{[\text{Cl}^-]_i} = \frac{[\text{Na}^+]_i}{[\text{Na}^+]_o} = 1.013
$

Now,

$E = -58.4 \log_{10} 1.013$

$= -0.33 \text{ mV.}$
APPENDIX D

ESTIMATE OF CHLORIDE SHIFT IN
RED CELLS SUSPENDED IN
RINGER SOLUTION
ESTIMATE OF CHLORIDE SHIFT IN RED CELLS
SUSPENDED IN RINGER SOLUTION

The chloride gained by the cells, if any, was estimated by measuring the chloride lost by the Ringer solution when a volume of packed cells was suspended in an equal volume of Ringer solution. The method of titration with AgNO₃ was used to measure the chloride concentration in solution.

(1) Preparation of 0.05M (50 mEq/L) AgNO₃:

AgNO₃ used for titration was prepared by dissolving 8.5 g AgNO₃ in 1000 ml distilled water. (M.W. = 169.98). The resultant solution had a calculated concentration of 0.05 molar, and contained 50 mEqAg/L.

When 5 ml (±0.1) of a sample was titrated every 1 ml of the prepared AgNO₃ required corresponded to a Cl⁻ concentration of 10 mEq/L.

(2) Preparation of Indicator:

Potassium chromate was dissolved in distilled water to give a yellow coloured solution. 10 drops of the indicator was added to 5 ml of the sample to be tested.
Titration was complete when the indicator turned light-brown. 1 drop (.05 ml) of the prepared AgNO₃ was enough to turn the colour of the indicator. Therefore, the inaccuracy caused by the use of the indicator was negligible.

(3) Titration of 0.1M (100 mEq/L Cl⁻) NaCl solution:
To test the AgNO₃ solution, 5 ml (±0.1) of a 0.1M NaCl solution was titrated with the AgNO₃. Results of 3 titrations showed 9.8, 10.0 and 10.1 ml of the AgNO₃ were used. A mean of 9.97 ml AgNO₃ used corresponded to 99.7 mEq/L Cl⁻ in the NaCl solution. This compared well to 100 mEq/L as calculated.

(4) Titration of Modified Isotonic Buffered Ringer Solution:
5 ml of the prepared Ringer solution was titrated with the prepared AgNO₃. Results of 8 titrations showed a range of 13.8 to 14.3 ml of AgNO₃ were used. A mean of 14.15 ± 0.17 (S.D.) was obtained. This corresponded to 141.5 ± 1.7 (S.D.) mEq/L Cl⁻ in the Ringer solution. Calculated value was 140 mEq/L. Results indicate that the method was consistent to within ±2 mEq/L Cl⁻ in the estimates.
Estimation of Cl\(^-\) Gained by Cells Suspended in Ringer solution:

15 ml (±0.1) of blood taken by venipuncture was centrifuged and as much as possible of the plasma was siphoned off and replaced by an equal, known volume of Ringer solution. The cells were completely dispersed and the sample centrifuged again. 5 ml (±0.1) of the supernatant was titrated. Assuming trapped plasma to be 5 to 10% when the blood was first centrifuged, we would have always over-estimated the Cl\(^-\) gained by the cells.

5 experiments were done, and the results showed that the Ringer after the washes to contain 138, 138, 140, 138, 142 mEq/L Cl\(^-\). Since the volume of Ringer solution used in each experiment was approximately equal to the volume of packed cells, even though we had likely over-estimated the Cl\(^-\) gain by the cells, the increase of intracellular Cl\(^-\) was only maximally 3 mEq/L cells, a figure which corresponded to the accuracy of the measurement.

On this basis, it was concluded that the intracellular Cl\(^-\) concentration did not change when the cells were suspended in Ringer solution which contained 140 mEq/L Cl\(^-\).
(6) Estimate of Cl⁻ Concentration in Plasma:

The concentrations of Cl⁻ in plasma were similarly studied. Two estimates were made. Results showed Cl⁻ concentrations of 110 and 115 mEq/L. Because plasma has a yellow colour even without the indicator, the sensitivity of the indicator was less in these experiments. Possible errors in these measurements might have been as high as ±5 mEq/L.
APPENDIX E

CALCULATION OF CHANGE IN LIQUID-JUNCTION POTENTIAL AS THE MICROELECTRODE TIP IS MOVED FROM RINGER SOLUTION TO VARIOUS MEDIA
LIQUID-JUNCTION POTENTIAL BETWEEN 3M-KCL AND VARIOUS MEDIA

The liquid-junction between liquids 1 and 2 can be calculated, if the conductances of the cations $U_1$, $U_2$ and anions $V_1$, $V_2$ respectively in both liquids are known, by the following equation:

$$E_J = \frac{RT}{F} \cdot \frac{(U_1 - V_1) - (U_2 - V_2)}{(U_1 + V_1) - (U_2 + V_2)} \cdot \ln \frac{(U_1 + V_1)}{(U_2 + V_2)}$$

The microelectrodes used in the experiments were always filled with 3M-KCl. If the junction potentials between 3M-KCl and various media were respectively calculated, then the change in the junction potential, as the microelectrode was moved from one medium to another, would be given by the algebraic difference between the two calculated junction potentials.

Our concern was mainly in the change when the microelectrode was moved from the Ringer solution to the cell interior. Since the Ringer solution was effectively an NaCl solution at a concentration of 0.11M, the junction potential between 3M-KCl and the Ringer solution could be accurately calculated, and was found to be -1.93mV.
Estimates of the junction potential between 3M-KCl and the red cell cytoplasm presented a problem since the exact conditions in the intact cell were not known. The limiting values could be calculated however, by assigning to the equation extreme values which could possibly exist in the cell.

Table 7 gives estimates of changes in the liquid-junction potential when the microelectrode is moved from Ringer solution to various media. In the table, columns $U_1$ and $V_1$ are constant at 150.0 and 160.0 millimho-cm$^{-1}$ respectively because phase 1 is 3M-KCl in the microelectrode in all cases. These values for the cation and anion conductances were calculated from the electrolyte conductance of 310.0 millimho-cm$^{-1}$, and the cation transfer number of 0.4872, obtained from Figures 30 and 31 for 3M-KCl at 25°C. These two figures were plotted from data given in the literature (Lange, 1952; MacInnes, 1961).

The total conductance of the red cell cytoplasm has been measured to be as high as 10.0 millimho-cm$^{-1}$ (Cook, 1952) and as low as 5.2 millimho-cm$^{-1}$ (Pauly & Schwan, 1966). Sections I, II and III of the calculations in the table consider the conductance of the cytoplasm to be 5.2, 7.5 and 10.0 millimho-cm$^{-1}$ respectively. These represent the two values from the available data, and an
**LEGEND TO TABLE**

$U_2/V_2$ - cation-to-anion conductance ratio.

$t^{25^\circ C}$ - cation transference number at 25°C.

$U_2+V_2$ - total ionic conductance.

$U_1$, $V_1$, $U_2$, $V_2$ - cation and anion conductances in 3M-KCl and in various media respectively.

$E_J$ - liquid-junction potential between 3M-KCl and various media.

$\Delta E_J$ - change in liquid-junction potential when microelectrode is moved from Ringer solution to various media.
<table>
<thead>
<tr>
<th>Ringer</th>
<th>0.62</th>
<th>0.3850</th>
<th>12.0</th>
<th>150.0</th>
<th>160.0</th>
<th>4.6</th>
<th>7.4</th>
<th>-1.98</th>
<th>0</th>
</tr>
</thead>
<tbody>
<tr>
<td>I A</td>
<td>0.96</td>
<td>0.4900</td>
<td>5.2</td>
<td>150.0</td>
<td>160.0</td>
<td>2.5</td>
<td>2.7</td>
<td>-3.31</td>
<td>-1.33</td>
</tr>
<tr>
<td>B</td>
<td>0.48</td>
<td>0.3250</td>
<td></td>
<td></td>
<td></td>
<td>1.7</td>
<td>3.5</td>
<td>-2.78</td>
<td>-0.80</td>
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<tr>
<td>C</td>
<td>0.24</td>
<td>0.1935</td>
<td></td>
<td></td>
<td></td>
<td>1.0</td>
<td>4.2</td>
<td>-2.30</td>
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<tr>
<td>D</td>
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<td>0.1070</td>
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<td>4.6</td>
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<tr>
<td>II A</td>
<td>0.96</td>
<td>0.4900</td>
<td>7.5</td>
<td>150.00</td>
<td>160.00</td>
<td>3.7</td>
<td>3.8</td>
<td>-3.10</td>
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<tr>
<td>B</td>
<td>0.48</td>
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<td></td>
<td></td>
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<td>2.4</td>
<td>5.1</td>
<td>-2.35</td>
<td>-0.37</td>
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<tr>
<td>C</td>
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<td>0.1935</td>
<td></td>
<td></td>
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<td>1.5</td>
<td>6.0</td>
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<td></td>
<td></td>
<td>0.8</td>
<td>6.7</td>
<td>-1.34</td>
<td>+0.65</td>
</tr>
<tr>
<td>III A</td>
<td>0.96</td>
<td>0.4900</td>
<td>10.0</td>
<td>150.0</td>
<td>160.0</td>
<td>4.9</td>
<td>5.1</td>
<td>-2.83</td>
<td>-0.85</td>
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<tr>
<td>B</td>
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<td>0.3250</td>
<td></td>
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<td></td>
<td>3.3</td>
<td>6.7</td>
<td>-1.91</td>
<td>-0.07</td>
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<tr>
<td>C</td>
<td>0.24</td>
<td>0.1935</td>
<td></td>
<td></td>
<td></td>
<td>1.9</td>
<td>8.1</td>
<td>-1.09</td>
<td>+0.89</td>
</tr>
<tr>
<td>D</td>
<td>0.12</td>
<td>0.1070</td>
<td></td>
<td></td>
<td></td>
<td>1.1</td>
<td>8.9</td>
<td>-0.64</td>
<td>+1.12</td>
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</table>
intermediate value. It is most likely, and is assumed here that the actual value falls within this range.

The red cell cytoplasm, except for the hemoglobin is essentially a solution of KCl at a concentration of approximately 0.1M. The cation transference number of 0.1M KCl at 25°C is about .4900 according to figure 33, and the corresponding cation-to-anion conductance ratio is 0.96. The presence of negatively charged hemoglobin at high concentration inside the cell will undoubtedly reduce the cation mobility and conductance. The conductance ratio will be proportionally reduced. It has been shown that a polyelectrolyte in solution will modify the ionic mobilities of K⁺ and Cl⁻ ions present in the solution. This is true especially for K⁺ ions where a reduction of mobility by as much as a factor of 4 has been reported (Overbeek, 1952, 1953).

In the table, line A in each group represents the situation where the hemoglobin has not altered the cation conductance, and the transference number used 0.4900 is that of 0.1M KCl at 25°C. Lines B, C and D in each group represent a 2, 4 and 8 fold reduction of the cation conductance respectively. The corresponding cation transference numbers are 0.3250, 0.1935 and 0.1070, and the corresponding cation-to-anion ratios are 0.48, 0.24 and 0.12. From the values of the total conductance
FIGURE 30

Relation between electrolyte conductance and concentration in NaCl and KCl solutions.
Relation between cation transference number and concentration in NaCl and KCl solutions.
(U_2 + V_2) and the conductance ratio, U_2/V_2, the values of U_2 and V_2 were determined. E_J, the liquid-junction potential between 3M-KCl and the various media were calculated from the equation, using appropriate values of U_2 and V_2 for each case. \( \Delta E_J \) is the change in liquid-junction potential when the microelectrode is moved from Ringer solution to the various media. E_J between 3M-KCl and Ringer solution is -1.98 mV. \( \Delta E_J \) was calculated by subtracting -1.98 from E_J.

It is doubtful that the cation conductance in the red cell cytoplasm has been reduced by less than a factor of 2, or more than a factor of 8. We consider that a reduction by a factor of between 4 and 6 would be reasonable. Moreover, we have chosen to take the intermediate value of conductance, i.e. 7.5 millimho-cm\(^{-1}\), as a reasonable estimate. Thus the likely values for \( \Delta E_J \) would be the ones in lines IIC and IID. The results of the estimate of the change in liquid-junction potential indicate that our measurements of the membrane potentials probably had been low, by up to about 0.5mV.