J. Membrane Biol. 1, 37–52 (1969)

The Cation Permeability of Erythrocytes in Low Ionic Strength Media of Various Tonicities*

JEROME A. DONLON and ASER ROTHSTEIN

Department of Radiation Biology and Biophysics, The University of Rochester, School of Medicine and Dentistry, Rochester, New York 14620

Received 21 February 1969

Summary. The steady state passive efflux of salt from human red blood cells was measured in various low ionic strength media in which the osmotic pressure ranged from 200 to 600 milliosmolar. Sucrose was used as the nonpenetrating nonelectrolyte. If the flux is plotted against the log of the salt concentration, the data for each tonicity can be fitted by three straight-line segments separated by two sharp inflections, one at low external salt concentrations (0.1 to 0.3 mM), confirming observations of LaCelle and Rothstein, and a second at higher salt concentrations (20 to 50 mM). As the osmolarity of the medium is increased, the inflection points shift systematically toward higher salt concentrations. The position of the inflection in every case seems to be uniquely determined by the membrane potential calculated from the Nernst equation with use of the chloride ratio. One inflection occurs at about 45 mV and the second at 170 mV in experiments at five different tonicities. Calculations from the Goldman equation suggest that the inflections represent potential-dependent changes to new permeability states. The osmotic pressure of the medium also influences the permeability. The coefficient is systematically reduced as the osmotic pressure is increased.

A rapid efflux of salt from human red blood cells suspended in a low ionic strength, isotonic sucrose medium has been reported by several investigators [7, 32, 33] since the first observations of Bang in 1909 [2]. Initially, a rapid acidification of the medium occurs, due primarily to an exchange of Cl^- for OH^- [16, 32] followed by a continuous loss of salt (primarily KCl). Since the permeability of red cells to anions is orders of magnitude greater than the permeability to cations [31], the anions can be assumed to be virtually at Donnan equilibrium and the membrane potential can be approximated by the Nernst equation with use of the chloride ratio (inside-to-outside concentration). Wilbrandt [33] suggested that this potential, together with the concentration gradient, constituted the driving force acting on the cations; on this basis he applied a simplified form of the Goldman flux equation to his results to calculate the cation

* Parts of this paper are included in "Passive Cation Efflux from Human Erythrocytes Suspended in Low Ionic Strength Media", Ph.D. thesis by Jerome A. Donlon, The University of Rochester, 1968.

permeability coefficient. He further speculated that the membrane potential may directly alter the cation permeability.

Recently, LaCelle and Rothstein [17] measured the steady state rate of cation efflux into media of constant but very low ionic strength by using a feedback arrangement based on conductivity. They observed that the cation flux plotted against the logarithm of the external salt concentration could be fitted by two straight lines with a sharp inflection point at about 0.2 mM salt. Using the simplified form of the Goldman equation proposed by Wilbrandt [33] for low external salt concentrations, they suggested that the inflection point represented a sharp transition in cation permeability. In the range above 0.2 mM salt, the permeability was constant and the change in salt efflux was attributed entirely to a change in the driving force. Below 0.2 mM, the permeability shifted to a new higher value with the transition completely reversible. The position of the inflection point was not directly dependent on external or internal pH or on the particular cation or anion pair in the medium, but it was markedly shifted toward higher ionic strength with an increase in temperature.

The conductivity-statting technique developed by LaCelle and Rothstein [17] is only practical at low salt concentrations (below 20 mM). For these reasons, their experiments were restricted to relatively low external salt concentrations, yet their data suggest that a second transition in permeability might occur at some salt concentration above 20 mM. Their line relating cation flux to external salt concentration extrapolated to zero flux at about 90 mM salt, yet at 90 mM a considerable driving force exists, and cells do lose salt under these conditions at rates considerably higher than are reported in the literature for cells in normal saline [17].

The present study was undertaken to extend the observations of LaCelle and Rothstein [17] on cation efflux from human erythrocytes to the full range of external salt concentrations from 0.10 to 150 mM, especially the unexplored region between 20 and 150 mM. The presence of a sharp and dramatic transition in permeability at about 0.2 mM salt was confirmed, and, in addition, a second transition was found at about 30 mM salt. The investigation then turned to the question of the factors involved in the sharp transitions and of the possible role of the membrane potential. The total electrochemical driving force acting on the cations and the membrane potential were manipulated, not only by changing the salt concentration of the medium but also by changing the salt concentration within the cells. The latter was accomplished by carrying out a series of measurements of cation efflux (and/or salt efflux) from cells suspended in media of various salt concentrations, with the tonicity at 200, 300, 400, 500, and 600 milli-

osmolar (controlled with sucrose). From the data, it was possible to conclude that dramatic changes in cation permeability occur at two discrete values of membrane potential. Conclusions could also be made concerning the effects of the osmolarity of the medium (with sucrose used as a non-penetrating solute).

Methods

Human blood stored 3 to 5 weeks in ACD at 4 °C was used in all experiments. The blood was washed (by alternate centrifugation, supernatant discard, and resuspension), once in isotonic Na-Tris buffer (pH 7.4), then twice in isotonic 0.95% NaCl, and finally in a 50/50 isotonic sucrose/NaCl solution. The washed cells were then resuspended to a hematocrit of about 50% in the isotonic sucrose-NaCl. For each experimental run the cells were resuspended in the appropriate sucrose-NaCl solution. At low salt concentration (below 20 mM), the passive cation efflux was determined by the steady state conductivity-statting technique described by LaCelle and Rothstein [17]. Briefly, the leakage of salt from the cells is detected by the increase in conductivity of the suspending medium. By a feedback system, sufficient salt-free sucrose solution is continuously added to the suspension to maintain a relatively constant conductivity, and thereby a relatively constant external salt concentration. The rate of salt efflux is determined from the slope of a graph of sucrose-addition against time. At salt concentrations above 20 mM, the salt leakage is slow and the changes in conductivity are so small that the signal-to-noise ratio becomes a limiting factor. The cation leakage was therefore determined in a nonsteady state system by analyzing the medium for changes in cation concentration, by using flame photometry. The two methods could be directly compared when the external salt concentration was in the range of 10 to 30 mM, and were found to give essentially the same results.

For the conductivity-statting procedure, the syringe drive used by LaCelle and Rothstein was replaced by Radiometer Autoburette (model ABU-1b) to permit a wider range of response. Conductivity calibration curves were predetermined for a range of sodium chloride (0.01 to 30 mM) in several sucrose solutions (200, 300, 400, 500, and 600 mM). Each set of experiments was run in a series such that the same washed blood was used for each sucrose solution studied, each estimate involving a 10- to 30-min run. The external pH was measured 2 min after mixing cells with the test solutions to allow the chloride-hydroxyl exchange to reach equilibrium. The pH values found were in the range 4.5 to 6.0 in which the cation efflux was shown to be almost independent of the pH of the medium [17]. The changes in pH during the course of an experiment were small (usually less than 0.1 unit). After the initial set of experiments, the pH was measured randomly rather than routinely. At the very low ionic strengths (< 1 mM NaCl), red cell clumping and rouleaux formation occurred, but this had no effect on the cation efflux rate and could be easily reversed by increasing the ionic strength. All experiments were performed at room temperature (23 °C). Cells to be used in the 200 milliosmolar solutions were first depleted of about one-third of their internal salt by allowing them to leak cation in a low salt medium for about 5 hr. When placed in hypotonic medium (200 mosm), the cells would return to their isotonic volume without hemolysis.

For the estimation of salt leakage by flame photometric analysis, the cell suspensions were allowed to leak for more than 5 hr without statting. Samples of the supernatant were taken periodically for analysis of Na^+ and K^+. Five-milliliter samples of washed blood cells were mixed in plastic vials with appropriate amounts of the sucrose-salt solutions to give a final isotonic hematocrit of about 15%. The sample vials were mixed by gentle shaking on a Dubonoff shaker. At the sampling time, 1- to 2-ml samples were taken from each vial and centrifuged in 4-ml glass tubes for 3 min in a lucite-head-modified Clay-Adams hematocrit

centrifuge. Samples were taken at 15 min, and 1, 2, 4, 7, and 10 hr. The pH was measured at 30 min. The potassium of the supernatant and the percent hemolysis at each point were measured. Hemolysis at 10 hr was almost always less than 2.0%. After a correction for the hemolysis, the rate of potassium efflux was calculated from the slope of the supernatant concentration against time.

Net sodium efflux into NaCl solutions could not be directly measured because of the small changes in sodium concentration that must be detected. In low ionic strength media (below 20 mM), the membrane shows little discrimination between Na^+ and K^+, and their effluxes are essentially proportional to their driving forces [17]. In the intermediate range of salt concentrations (20 to 100 mM), a series of experiments was performed in a choline chloride medium allowing a direct measure of both sodium and potassium effluxes. The possible contribution of active transport was tested by determining the effect of ouabain (0.14 mM) on cation efflux.

Measurements of anion distribution have not been made for cells suspended in low electrolyte media of different tonicities as used in the present experiments. Therefore, chloride distributions were measured isotopically[1], using ^{36}Cl with a specific activity of 0.46 μc/ml added to sucrose-salt solutions of four salt concentrations (4, 10, 20, and 50 mM), at each of four osmolarities (300, 400, 500, and 600 mosm). Washed blood was added to these solutions to an isotonic hematocrit of about 20%. The suspensions were mixed and allowed to equilibrate for 10 min. The tubes were then centrifuged for 20 min at 4,000 rpm. Next, 1 ml of supernatant and 0.5 ml of packed blood cells each were pipetted onto 1-inch diameter counting planchets. The remaining blood cells were hemolyzed in 20 ml of deionized water, and a 1-ml sample of the hemolysate was placed on a planchet. The sample planchets and appropriate standards were dried and counted. After corrections were made for self-absorption, dilutions, and background, the chloride distribution ratios were determined and the Donnan chloride potential was calculated.

Several titration curves for human hemoglobin have been reported [11, 14, 29], but none have been reported at the ionic strengths that would exist in cells suspended in sucrose media as high as 600 mosm. Therefore, cells were hemolyzed by brief sonication (10 sec with a Bronson Sonicator), and titrations of the hemolysates were performed automatically using a Radiometer titration apparatus (model TTT 1) over the pH range 5.5 to 8.5 at 23 °C, at ionic strengths of 0.15 to 0.30 and at two hemoglobin concentrations (1.0 and 3.8 mM).

The osmotic response of red cells in hypertonic sucrose-salt medium was measured over a range of tonicity from 300 to 800 mosm (including 50 mM salt). A control solution of 150 mM NaCl was also used. The blood was suspended in the various test solutions to an isotonic hematocrit of about 30%. Extracellular space in centrifuged red cells was determined by ^{131}I Albumin (0.20 μc/ml specific activity) distribution. The osmotic shrinkage was measured by hematocrit with a correction for extracellular space. In a parallel sample the internal cations were also measured, after lysis of the packed cells, by flame photometry with appropriate correction for extracellular space.

Cell water was determined gravimetrically after a 1-ml aliquot of blood was dried for 48 hr at 95 °C. The osmolalities of solutions were measured on a Fiske Freezing Point Depression Osmometer (Model G-62). Solutions of 200, 300, 400, and 600 milliosmolar gave milliosmolalities of 205 ± 5, 308 ± 12, 412 ± 15, 528 ± 18, and 640 ± 21. In this paper, solutions are referred to as milliosmolar (mosm). Hemolysis was measured by the spectrophotometric (540 mμ) determination of released hemoglobin, using the Cyanmethemoglobin procedure [8].

1 Each sample was counted three times to a present count of 10,000 in a Geiger tube, with a Nuclear Chicago 181-A scaler and 110-B automatic sample changer. Counter efficiency for ^{36}Cl was 7.5%.

Assumptions and Equations

The basic assumptions and equations on which the calculations of the membrane potential and chemical gradient are based included the following.

(a) Anions are in Donnan equilibrium [5, 9]

$$\frac{\gamma_i \mathrm{Cl}_i}{\gamma_0 \mathrm{Cl}_0} = \frac{\mathrm{OH}_i}{\mathrm{OH}_0} = \frac{H_0}{H_i} = r \tag{1}$$

where γ is the activity coefficient. The activity coefficients used for chloride are the mean molal activity coefficients of sodium chloride at 25 °C at the given concentration, with data taken from the tables in Robinson and Stokes [24]. Although Donnan distributions for anions have been reported in the literature for a wide variety of conditions [5, 9], there is no information at the tonicities and low external salt concentrations used in the present experiments. Determinations of ^{36}Cl distributions were made at all tonicities (Table 1). The chloride potential determined from the isotope ratio and that calculated independently from pH_0 and Eq. (5) are in good agreement, indicating that the Cl^- is indeed in Donnan equilibrium under the conditions of the present experiments.

Table 1. *Comparison of the membrane potentials calculated from the chloride distribution ratio and from other assumptions* [Eq. (5)]

Osmolarity-salt (mOsm-mM)	From chloride distribution		From assumptions		Ratio[a]
	Donnan ratio	potential (mV)	Donnan ratio	potential (mV)	
300 – 5	18.6	74.9	24.6	82.0	0.91
400 – 5	27.3	84.7	31.6	88.5	0.96
500 – 5	36.6	92.2	38.0	93.2	0.99
600 – 5	47.1	98.7	46.0	98.0	1.01
300 – 10	11.7	63.1	12.2	64.0	0.99
400 – 10	15.1	69.5	15.4	70.0	0.99
500 – 10	20.2	77.0	18.8	75.2	1.02
600 – 10	24.8	82.3	22.8	80.0	1.03
300 – 20	7.4	51.2	6.7	49.0	1.05
400 – 20	11.3	62.1	8.7	55.5	1.12
500 – 20	15.4	70.1	10.5	60.0	1.17
600 – 20	16.6	72.0	12.6	65.0	1.11
300 – 50	2.4	22.5	3.0	28.0	0.80
400 – 50	3.2	30.0	3.8	34.0	0.88
600 – 50	5.2	43.0	5.6	44.0	0.97

[a] $\bar{x} = 1.00 \pm 0.03$.

(b) Electroneutrality exists inside the red blood cell, and chloride is the major internal anion [9]

$$C_i = \mathrm{Cl}_i + \mathrm{Hgb} \tag{2}$$

where C_i is the total internal cation concentration, and Hgb is the charge contribution from internal dissociated nondiffusible anions (primarily hemoglobin).

(c) The dissociation curve for the cellular contents is essentially log linear from the isoelectric point [14] within the range of internal pH used in the experiments so that the internal buffering capacity could be readily calculated.

$$\text{Hgb} = B(\text{pH}_i - I) \tag{3}$$

where B is the buffering capacity in meq/liter red blood cell (RBC) per pH unit, and I is the isoelectric point. Titration curves of hemolysates and of human hemoglobin have been reported in the literature but not under conditions that might exist in the cell in the present experiments. German and Wyman [11] gave values of 6.81 for the isoelectric point and 61.0 meq/liter of cells per pH unit for the buffering capacity of human oxyhemoglobin. The data of Harris and Maizels [14] on human hemolysate titrations yielded an isoelectric point of 6.80 and a buffer capacity of 50.0 meq/liter RBC per pH unit. An isoelectric point of 7.15 and a buffer capacity of 45.0 meq/liter RBC per pH unit have also been reported for oxyhemoglobin [29]. Our hemolysate titration curves indicated essentially no effect of ionic strength (0.15 to 0.30) or of hemoglobin concentration (1.0 and 3.8 mM) on the buffer capacity for pH values near the isoelectric point. The curves were log linear over the pH range of 6.2 to 7.6 and gave a buffer capacity of 62.0 meq/liter RBC per pH unit. An average isoelectric pH of 6.92 was chosen from the values reported in the literature [11, 14, 29].

(d) The cells are always in osmotic equilibrium and the osmotic responses to sucrose follow the van't Hoff relation [22, 25]:

$$\pi_0(V_0 - b) = \pi(V - b) \tag{4}$$

π_0 is the isotonic osmotic pressure; V_0 is the isotonic cell volume; and b is the nonosmotic volume.

The results of our experiments on the osmotic shrinkage of red cells suspended in unbuffered hypertonic sucrose-salt media indicated that all the cell water was osmotically active and that the cell shrinkage followed a van't Hoff relation.

Recently, Cook [6] has suggested that all of the red cell water is osmotically active. Other studies also support this assumption [10].

If the total cation content remains constant during cell shrinkage, then the internal cation concentration, C_i, can be calculated from a simple osmotic relationship: $C_i = C_{i0}\frac{\pi}{\pi_0}$, where C_{i0} is the isotonic internal cation concentration.

The cell water of blood-bank cells suspended in unbuffered medium was determined to be $62.7 \pm 0.7\%$ of cell volume (three samples), and the total internal cation concentration of 10 blood-bank samples was 152.0 ± 4.5 mmoles/liter cell water. The potassium and sodium concentrations were 110.5 ± 4.9 mmoles/liter cell water and 41.5 ± 3.8 mmoles/liter cell water, respectively.

(e) Combining Eqs. (2) and (3) for Hgb and then substituting for Cl_i in Eq. (1) yields, upon rearrangement, the following result:

$$H_0\,\text{Cl}_0 = H_i\frac{\gamma_i\,\text{Cl}_i}{\gamma_0} = B \cdot H_i\frac{\gamma_i}{\gamma_0}\left[\left(\frac{C_i}{B} + I\right) - \text{pH}_i\right]. \tag{5}$$

This is a transcendental equation for H_i. Given H_0, Cl_0, C_i, B, and I, the equation was solved for H_i by an iterative method on an IBM Systems 360 digital computer. Using H_i, the Donnan ratio (r) was determined, and then the membrane chloride potential was calculated from the Nernst equation, $E = \frac{RT}{F}\ln r$, where R, T, and F have their usual meaning.

(f) The membrane mobilities of sodium and potassium are essentially equal. This was shown to be true by LaCelle and Rothstein [17] for passive efflux into low external salt concentrations. The data were extended to the intermediate salt range (25 to 75 mM) by suspending the cells in choline chloride medium and measuring the rate of appearance of Na^+ and K^+ by flame photometry. The results, shown in Table 2, although somewhat scattered, indicate no appreciable discrimination between Na^+ and K^+.

Table 2. *Passive potassium and sodium effluxes into choline chloride medium*[a]

Osmolarity (mosm)	Ch Cl (mM)	K efflux mmoles/liter RBC/hr	Na efflux mmoles/liter RBC/hr	Mobility ratio (U_k/U_{Na})[b]
300	24	0.14	0.12	0.93
300	48	0.13	0.06	1.32
300	72	0.10	0.05	0.75
600	48	0.49	0.40	0.94
600	72	0.38	0.28	0.92

[a] The internal potassium and sodium concentrations are 77.5 mM K^+, 63.5 mM Na^+, 158 mM K^+, and 130 mM Na^+ for the 300 and 600 milliosmolarities, respectively.

[b] Each mobility was calculated as efflux per unit electrochemical driving force. $\bar{x} = 0.97 \pm 0.33$.

(g) The efflux of salt is a passive efflux with no appreciable active transport component opposing it. In the very low external salt range, the salt efflux observed by LaCelle and Rothstein [17] was of such a magnitude that the pump component, if any, was overwhelmed. However, in the high salt range in which the efflux rates are relatively lower, the pump flux, which can be as high as 2 mmoles/liter RBC per hr [26], must be considered. The activity of the pump was minimized in several ways. The experiments were carried out at 23 °C (rather than at 37 °C) which would markedly reduce the pump flux. The efflux was measured into initially potassium-free medium, and the K^+ concentration was always well below 2 mM, the K_m for the pump [26]. Three-week-old blood-bank blood is depleted in its cellular ATP to about 20% of normal [21]. Finally, two experiments at 48 mM were carried out in the presence and absence of 0.14 mM ouabain, and no differences in effluxes were observed.

Results

The steady state cation effluxes at various external salt concentrations are shown in Fig. 1 for two representative osmolarities (300 and 600 mosm). The data encompass a wide range of external salt concentrations (0.1 to 100 mM) and of passive effluxes (0.5 to 100 mmole/liter RBC per hr). The curves for the five osmolarities studied are shown in Fig. 2, with the data points omitted for the sake of clarity. In each case, the data on semi-log plot can be fitted by three straight-line segments with two inflection points. One of these, at 0.2 mM (300 mosm), has previously been described by LaCelle and Rothstein [17]. The others occurring at higher salt concentrations (20 to 50 mM) are reported for the first time. With increasing

tonicity, the pattern is not changed, but the inflection points are moved toward higher fluxes and toward higher salt concentrations. The data points determining the two slopes at the lower salt concentrations (below 20 to 50 mM) in Figs. 1 and 2 are numerous and reproducible. The fluxes are relatively high, and the experimental procedure of conductivity statting is quite precise. The lines, determined by the method of least squares, can

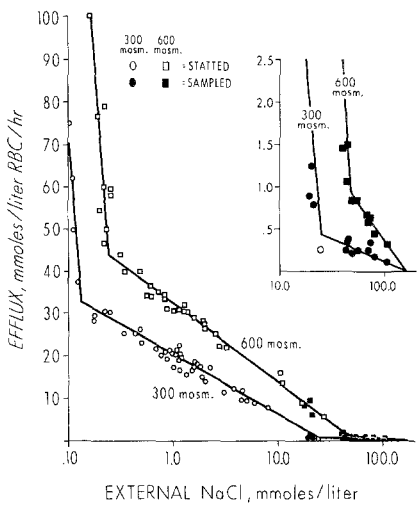

Fig. 1

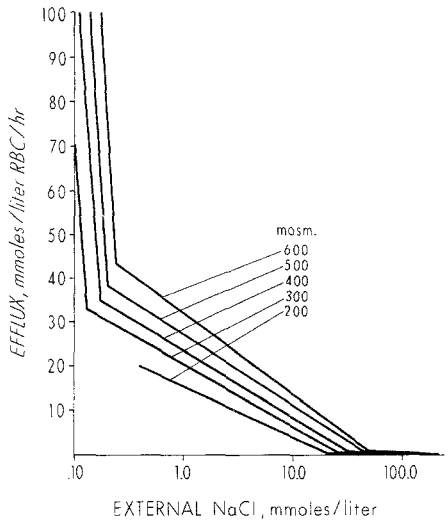

Fig. 2

Fig. 1. The effect of external NaCl concentration on the rate of salt efflux from red cells suspended in 300 and 600 mOsm media. Closed symbols represent data from the nonsteady state experiments. An enlargement of the region of higher ionic strength is shown is the inset

Fig. 2. The effect of external NaCl concentration and osmolarity of the medium on the rate of salt efflux from red cells. Each curve was determined by at least 30 data points

be precisely located. The data points for the line to the right (at high salt concentrations) show more scatter (see inset of Fig. 1 for expanded scale). The fluxes are lower and the procedure by sampling and analysis of the medium is inherently less accurate. Nevertheless, because the difference in slopes is so great (about 30-fold), the inflection point can be accurately determined. The two experimental procedures overlap in the concentration range 10.0 to 30.0 mM with good agreement.

Factors which might influence the position of the inflection points of Fig. 2 include internal and external pH, salt concentrations, chemical gradient across the medium, and the membrane potential. The latter was calculated from the Nernst chloride potential according to the assumptions and equations previously discussed. In Fig. 3, this calculated poten-

tial is plotted against the cation fluxes for the data at each tonicity. The inflections all occur at particular membrane potentials, about 45 and 170 mV. The uniqueness of the membrane potential as the controlling parameter of passive efflux permeability is further demonstrated in Table 3, where the salt concentrations, pH values, potentials, electrochemical gradients, and fluxes are given for each inflection point. The internal salt

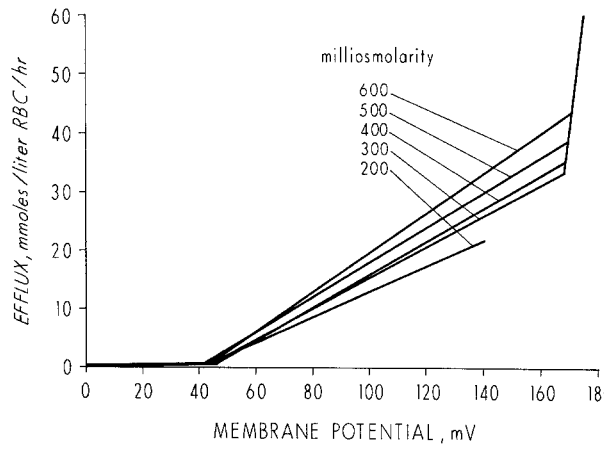

Fig. 3. The effect of the membrane potentials on salt efflux into low ionic strength media of various tonicities

concentration is virtually proportional to the osmolarity and thus varies by a factor of three. The external salt concentration varies by a factor of two; the chemical gradient ($C_I - C_0$) varies by a factor of about three; the total electrochemical gradient (*see* Results for details of calculation) varies

Table 3. *Properties of the inflection points*[a]

Osm	NaCl	pH_0	pH_i	E	DF	Flux
300	0.13	4.65	7.55	167	1,000	33.0
400	0.17	4.70	7.57	168	1,340	35.0
500	0.20	4.75	7.70	169	1,712	38.5
600	0.24	4.80	7.74	170	2,068	43.5
200	20.5	5.85	6.58	42.6	202	0.30
300	25.0	5.87	6.64	45.0	315	0.44
400	31.0	5.90	6.68	45.8	427	0.66
500	45.0	5.95	6.67	42.1	507	0.70
600	47.5	5.93	6.70	44.8	640	0.94

[a] Abbreviations are: Osm, osmolarity, milliosmolar; NaCl, external salt concentration, mmoles/liter; E, membrane potential, mV; DF, electrochemical driving force, mmoles/liter; and Flux, cation efflux, mmoles/liter RBC per hr.

two- to threefold; but the potential varies by less than 7% in one case and less than 2% in the other. Another possible factor is pH. In the present experiments, the range of internal and external pH values at the two inflection points were relatively small, but LaCelle and Rothstein [17] explored a wide range of pH values and found no shift in the 0.2 mM inflection point.

From the data, it is possible to calculate permeability coefficients by using the Goldman equation [12]. One of the limitations on its use is the constant field assumption for the electrical gradient. Recently, however, Barr [3] has suggested that this restriction may be relaxed in certain special cases. One case is the membrane whose permeability to anions is much greater than the permeability to cations so that the anions are in electrochemical equilibrium, a condition that is applicable to the red blood cell membrane. The integrated form of the Goldman flux equation can be written as:

$$M_i = \frac{U_i}{a} \frac{RT}{F} \ln r \left[\frac{r C_i^I - C_i^0}{r-1} \right] \tag{6}$$

where M_i is flux of i^{th} ion, moles/cm^2 per sec; u_i, mobility of i^{th} ion, cm/sec per V/cm; a, membrane thickness, cm; r, Donnan ratio; C_i^I, internal concentration of i^{th} ion, moles/liter; C_i^0, external concentration of i^{th} ion, moles/liter; and R, T, F, usual meanings.

At low external salt concentrations, the Donnan ratio (r) is large such that $r \gg 1$ and $r C_i^I \gg C_i^0$. In this way, the Eq. simplifies to the form used by Wilbrandt (33) and by LaCelle and Rothstein (17):

$$M_i = \frac{U_{i\,RT}}{a\,F} \ln r \cdot C_i^I. \tag{7}$$

However, in this present study, the complete Goldman equation [Eq. (6)] was applied, and the computations were performed on an IBM Systems 360 digital computer. Because the membrane mobilities of sodium and potassium are about equal (see section on Assumptions), the Goldman equation can be applied to a total cation efflux driven by a total cation driving force. From Eq. (6) the electrochemical driving force (DF) is considered to be:

$$\text{DF} = \ln r \left(\frac{r C^I - C^0}{r-1} \right) \quad \text{moles/liter}.$$

The permeability coefficient is therefore:

$$P = \frac{U}{a} \frac{RT}{F} \quad \text{cm/sec}.$$

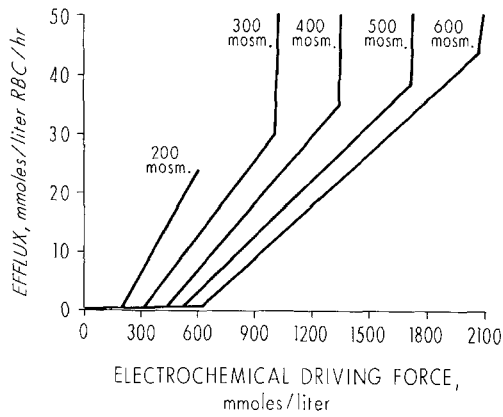

Fig. 4. The effect of the electrochemical driving force on salt efflux from red cells in low ionic strength media of various tonicities

In Fig. 4, the measured fluxes are plotted against the calculated driving forces. As in the graphs of flux versus log of salt concentration (Figs. 1 and 2), the data for each osmolarity can be fitted by three straight-line segments with two sharp inflection points. The slopes for the three segments are approximately in the ratio of 1:30:300 in each case, with some shift to the right as the osmolarity is increased. That is, for a given driving force, the flux is decreased with increased osmolarity, suggesting that the permeability is reduced as the osmolarity is increased.

The Goldman equation predicts a straight-line relationship between electrochemical driving force and flux. On this basis, a permeability coefficient can be calculated for each straight-line segment in Fig. 4, if one assumes that a sharp change in permeability occurs at each inflection point. Such a calculation would, however, be strictly valid only for the left-hand segment that goes through the origin, for the Goldman equation predicts that the flux will be zero at a zero driving force (that the line relating flux and driving force must go through the origin). If new permeability channels were opened up at 45 mV (Fig. 3), then the flux should suddenly jump to a much higher value because the driving force at 45 mV is considerable. At higher potentials, the flux should follow a slope that would extrapolate to the origin. If all-or-none increases in permeability occur at 45 and 170 mV, the Goldman equation can be applied only to the slopes provided that the intercepts of the straight-line segments of Fig. 4 represent zero driving force at the site of the permeability change, even though the driving force across the whole membrane is considerable.

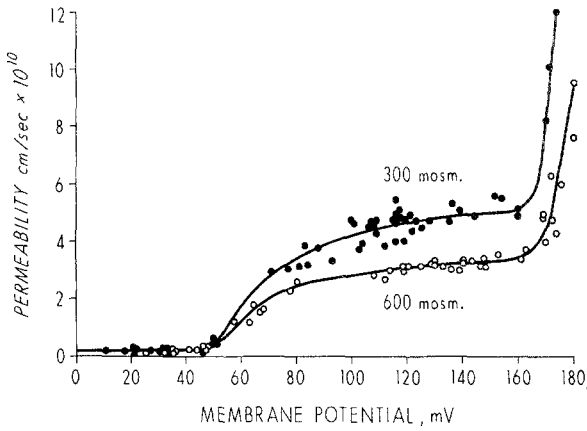

Fig. 5. The relationship of the membrane potential (calculated from the Cl ratio) on the permeability coefficient of red cells suspended in low ionic strength media at two tonicities, 300 and 600 mOsm

Such a specific partition of driving forces across the membrane is possible, but it seems unlikely.

In attempting to gain further insight into the nature of the potential-dependent changes in flux of Fig. 3, each datum was recalculated in terms of a permeability coefficient by using the Goldman equation. Two representative sets of data (for 300 and 600 mosm media) are shown in Fig. 5. In the range of potentials from 0 to 45 mV, the average permeability coefficient for the data from experiments to all five osmolarities was 0.22 ± 0.061 cm/sec based on 31 estimates. In this range of potentials, the permeability is independent of the potential. A decrease in permeability was noted as the osmolarity of the medium was increased, but the relationship was not statistically significant ($P > 0.05$). With potentials above 45 mV, the calculated permeability coefficient increases gradually toward another plateau, relative independent of potential between 105 to 170 mV. Then it rises sharply again above 170 mV. The curves for the other tonicities (200, 400, and 500 mosm) follow the same pattern.

The potential-dependent permeability change is very large. The values on the plateau (at 120 mV) range from 3.1 to 5.8×10^{-10} cm/sec compared to 0.22×10^{-10} cm/sec at the base line (between 0 and 45 mV), an increase of 14- to 25-fold. On the plateau, a consistent relationship between the permeability and the osmolarity of the medium was found, with permeability decreasing with increasing osmolarity. In Fig. 6, the permeability data are plotted against the reciprocal of the osmotic pressure, in the form of the van't Hoff equation. The points fall along a straight line

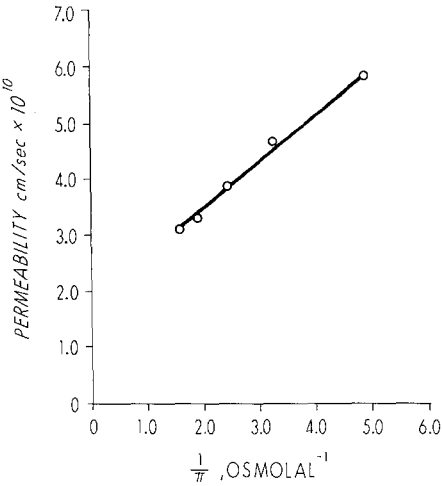

Fig. 6. The effect of external osmolarity on the cation permeability coefficient of red blood cells in low ionic strength media. The permeabilities at a membrane potential of 120 mV were chosen from data curves similar to those of Fig. 5

which suggests that the decrease in permeability may be related to the dehydration of the membrane and to the shrinking that may take place in hypertonic media.

Discussion

The data presented in this paper clearly indicate that large changes in the behavior of the red cell membrane toward cation efflux occurs at two specific membrane potentials, 45 and 170 mV (outside positive). Although the relationship of cation flux to potential shows very sharp inflections, suggesting essentially an all-or-none shift from one membrane state to another, the simplest model that fits the data would suggest a gradual transition from one state to another (Fig. 5). The model assumes that the cation permeability can be described by the Goldman equation. With low potentials (up to 45 mV) imposed on the membrane, the permeability is not affected, but at 45 mV (one of the sharp inflection points of the flux data of Figs. 1–4) a perceptible increase in permeability occurs. Further increase in potential is associated with further increases in permeability with a tendency toward a maximum represented by the plateau above 100 mV. A second increase in permeability is initiated at 170 mV, but the data do not extend far anough to ascertain whether or not a new stable state is reached at very high potentials. The increases in permeability at high potentials may represent the behavior of the "normal" permeability channels or the opening up of new channels. The gradual increase may represent the thresholds of a population of sites or the gradual opening of all of the sites under the force exerted by the potential gradient.

Many investigators have concerned themselves with potential-dependent transitions in the membrane, particularly with respect to depolarization phenomena in nerve [27, 28]. These transitions, however, occur as the potential is increased from outside negative toward zero, whereas the changes reported here occur as the potential is raised from zero in the direction of increasing outside positive. Reversible potential-dependent changes in the membrane probably represent rearrangements of charged components, particularly dipolar elements [30]. Artificial bilipid membranes subjected to transmembrane voltages of about 160 mV will rupture [19]. The electric field within the membrane at these voltages is about 2×10^5 V/cm. The electric field within the red cell membrane at the second inflection (45 mV) is comparable to 0.5×10^5 V/cm, but the effects are reversible [17]. Perhaps more germane are experiments on the electrical excitability of "black" phospholipid membranes treated with proteins including acetone powders of human red cell ghosts [19, 20]. The threshold potential is in the range of 40 to 50 mV, the same as that for the lower threshold in the present study.

Several authors have speculated on the possibility of a strong electric field inducing a confirurational change in membrane components [1, 4, 15]. Hill [15] has pointed out that a small change in electric field can trigger a polyelectrolyte contraction. Therefore, he suggests it may be possible for an electric field to trigger a membrane phase transition via a ligand "contraction". Bass and Moore [4], in discussing the electric fields in perfused nerve, point out that the electric field does not have to be uniform throughout the membrane when internal salt is diluted. This could lead to a dielectrophoretic effect in which macroscopic movements of dielectric material to a region of high field intensity would occur. Furthermore, they suggest that changes in the electric field will produce a Wien dissociation effect on a weak acid-base equilibrium, causing an altered membrane pH of 0.1 to 0.2 pH units; this in turn will be responsible for the configurational change leading to permeability changes. In the present case, however, the fact that the transitions are independent of internal and external pH would not support such a mechanism. In more theoretical terms, Agin [1] has also suggested that these effects of high electric fields dserve more consideration in membrane biophysics.

Several molecular models of nerve excitation have been presented in the literature [13, 18, 27, 28]. Goldman [13] has suggested that the dipoles of certain phospholipids can change their orientation and cation combining properties under the influence of an electric field. The phosphoryl groups act as ion exchange "gates" and are considered to be in one of

three configurational states, favorable for either calcium, sodium, or potassium binding. Hill [15] has presented a brief thermodynamic analysis of the cooperativity in biological membranes. He assumes that the surface of the membrane is a two-dimensional lattice of dipoles, and he computes the theoretical partition function for two configurational states under the influence of thermal and electrical energy. In this regard, it is of some interest that the only other factor besides potential that is known to influence the inflections in red cell permeability is the temperature. Thus, LaCelle and Rothstein [17] found the inflections to shift toward higher salt concentrations with increased temperature. Recalculation of their data in terms of the potential at which the inflections occur indicates a continuous linear decrease from 190 to 105 mV as the temperature is increased from 14 to 37 °C. This relationship indicates the possibility of an energy threshold requirement for the permeability changes.

The effect of the osmolarity of the medium on the cation permeability (Fig. 6) is perhaps related to the recent observations of Rich et al. [23] on water permeability in red cells. Their finding of a decreased hydraulic conductivity with increasing osmolarity is in concert with the present findings on passive cation permeability. Their data are also consistent with the effect being controlled by dehydration of the membrane.

This work was performed under contract with the U.S. Atomic Energy Commision at The University of Rochester Atomic Energy Project, Rochester, N.Y. It has been assigned Report No. UR-49-1056.

We wish to thank Dr. David A. Goldstein and Mr. Philip A. Knauf for their suggestions concerning the theoretical aspects of the paper.

References

1. Agin, D. 1967. Electroneutrality and electrodiffusion in the squid axon. *Proc. Nat. Acad. Sci., Wash.* **57**:1232.
2. Bang, J. 1909. Physiko-chemische Verhältnisse der Blutkörperchen. *Biochem. Z.* **16**:255.
3. Barr, L. 1965. Membrane potential profiles and the Goldman equation. *J. Theoret. Biol.* **9**:351.
4. Bass, L., and W.J. Moore. 1967. Electric fields in perfused nerves. *Nature* **214**:393.
5. Bromberg, P.A., J. Theodore, E. Robin, and W. Jensen. 1965. Anion and hydrogen ion distribution in human blood. *J. Lab. Clin. Med.* **66**:464.
6. Cook, J. S. 1967. Nonsolvent water in human erythrocytes. *J. Gen. Physiol.* **50**:1311.
7. Davson, H. 1939. Studies on the permeability of erythrocytes. VI. The effect of reducing the salt content of the medium surrounding the cell. *Biochem. J.* **33**:389.
8. Drabkin, D.L. 1945. Hemoglobin, glucose oxygen and water in the erythrocyte. *Science* **101**:445.
9. Funder, J., and J.O. Wieth. 1966. Chloride and hydrogen ion distribution between human red cells and plasma. *Acta Physiol. Scand.* **68**:234.
10. Gary-Bobo, C.M. 1967. Nonsolvent water in human erythrocytes and hemoglobin solutions. *J. Gen. Physiol.* **50**:2547.

11. German, B., and J. Wyman. 1937. Titration curves of hemoglobin. *J. Biol. Chem.* **117**:534.
12. Goldman, D.E. 1943. Potential, impedance, and rectification in membranes. *J. Gen. Physiol.* **27**:37.
13. – 1964. A molecular structural basis for the excitation properties of axons. *Biophys. J.* **4**:167.
14. Harris, E.J., and M. Maizels. 1952. Distribution of ions in suspensions of human erythrocytes. *J. Physiol.* **118**:40.
15. Hill, T.L. 1967. Electric fields and the cooperativity of biological membranes. *Proc. Nat. Acad. Sci., Wash.* **58**:111.
16. Jacobs, M., and A.K. Parpart. 1932. Osmotic properties of the erythrocyte. V. The rate of hemolysis in hypotonic solutions of electrolytes. *Biol. Bull.* **63**:224.
17. LaCelle, P., and A. Rothstein. 1966. The passive permeability of the red blood cell to cations. *J. Gen. Physiol.* **50**:171.
18. Lettvin, J.V., W.F. Packard, W.S. McCulloch, and W. Pitts. 1964. A theory of passive ion flux through axon membrane. *Nature* **202**:1338.
19. Mueller, P., and D.O. Rudin. 1963. Induced excitability in reconstituted cell membrane structures. *J. Theoret. Biol.* **4**:268.
20. – – 1967. Action potential phenomena in experimental bimolecular lipid membranes. *Nature* **213**:603.
21. Nakao, M., T. Nakao, S. Yamazoe, and H. Yoshikawa. 1961. Adenosine triphosphate and shape of erythrocytes. *J. Biochem.* **49**:487.
22. Ponder, E. 1943. The osmotic behavior of crenated red cells. *J. Gen. Physiol.* **27**:273.
23. Rich, G.T., R.I. Sha'afi, A. Romualdez, and A.K. Solomon. 1968. Effects of osmolality on the hydraulic permeability coefficient of red cells. *J. Gen. Physiol.* **52**:941.
24. Robinson, R.A., and R.H. Stokes. 1959. Electrolyte Solutions. 2nd rev. edition. 492. London: Butterworth and Co.
25. Savitz, D., V.W. Sidel, and A.K. Solomon. 1964. Osmotic properties of human red cells. *J. Gen. Physiol.* **48**:79.
26. Stein, W.D. 1967. The Movement of Molecules Across Cell Membrane. p. 225. Academic Press Inc., New York.
27. Tasaki, I., and I. Singer. 1966. Membrane macromolecules and nerve excitability: A physico-chemical interpretation of excitation in squid giant axons. *Ann. N.Y. Acad. Sci.* **137**:792.
28. Tobias, J.M. 1964. A chemically specified molecular mechanism underlying excitation in nerve: A hypothesis. *Nature* **203**:13.
29. Vodrážka, Z., and J. Čejka. 1961. Interaction of human hemoglobin with hydrogen ion. *Biochim. Biophys. Acta* **49**:502.
30. Wei, L.Y. 1969. Role of surface dipoles on axon membrane. *Science* **163**:280.
31. Whittam, R. 1964. Transport and Diffusion in Red Blood Cells. p. 76. Williams and Wilkins Co., Baltimore.
32. Wilbrandt, W. 1940. Die Ionpermeabilität der Erythrocyten in Nichtleiterlösungen. *Arch. ges. Physiol.* **243**:537.
33. –, and H.J. Schatzmann. 1960. Changes in the passive cation permeability of erythrocytes in low electrolyte media, p. 340. *In* Ciba Foundation Study Group Symposium No. 5. Regulation of the Inorganic Ion Content of Cells. Churchill, London.