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ERYTHROCYTE MEMBRANE POTENTIALS DETERMINED BY HYDROGEN ION DISTRIBUTION

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Summary

If the extracellular fluid is left unbuffered, dynamic membrane potential changes in the red blood cell may be determined from external pH readings. For some types of experiments it is necessary to accelerate H^+ equilibration by adding minute amounts of hydrogen carriers. The method is independent of hematocrit over a wide range of membrane potential changes. Membrane potential jumps produced by permeability changes or by changes in ionic composition may be measured. The method provides a convenient means of measuring parameters of both the conductive and non-conductive anion pathways in the red cell.

Introduction

In principle, the membrane potential of a cell can be determined from the distribution of any ion at equilibrium. This principle has been widely used in cells too small for the convenient introduction of a microelectrode. In the case of normal red blood cells Cl^- appear to be in a Donnan equilibrium [1,2] and as a result it has become standard practice to estimate red cell membrane potentials from Cl^- distribution. Although this procedure is suitable during steady states, it is not always applicable under dynamic conditions, e.g. when K^+ permeability is suddenly increased [3]. This limitation has led to the introduction of fluorescent membrane probes whose intensity of fluorescence parallels changes in membrane potential [4].

In this paper, we propose an alternative which relies on H^+ distribution [5]. Like Cl^-, the distribution of H^+ follows the predictions of a Donnan equilibrium but its equilibration under more dynamic conditions may be too slow to

Abbreviations: CCCP, carbonyl cyanide *m*-chlorophenyl hydrazone; TCS, 3,3′,4′,5-tetrachlorosalicylanilide; SITS, 4-acetamido-4′-isothiocyano-2,2′-disulfonic stilbene; DIDS, 4,4′-diisothiocyano-2,2′-disulfonic stilbene; HEPES, *N*-2-hydroxyethylpiperazine-*N*′-2-ethane sulfonic acid.

follow changes in membrane potential. However, in contrast to Cl^-, there are a number of agents which, acting as H^+ carriers [6], increase the hydrogen permeability of natural and artificial membranes by several orders of magnitude. We shall show that use of small doses of these agents accelerates H^+ equilibration sufficiently to follow rapid membrane potential changes which occur during the Gardos effect [7—9], when valinomycin is added to the cells, and when anion composition of the medium is suddenly changed [10].

Materials and Methods

Blood. Blood from human donors was collected using acid/citrate/dextrose solution or heparin as an anti-coagulant. After centrifugation at 3000 × *g* and removal of the serum and buffy coat the cells were washed three times in isotonic unbuffered saline or sucrose solutions. The ionic composition of the saline depended upon the type of experiment for which the blood was intended and is given in the captions of the figures. For some experiments the pH of the blood suspension was adjusted by adding small amounts of NaOH or HCl.

For experiments in which K^+ was measured the cells were suspended in 150 mM choline chloride. After pH adjustment to 7.4 they were washed twice more in choline chloride to remove residual external K^+. 6-ml aliquots were then centrifuged and the supernatant was removed. Packed cells were stored at 5°C and were resuspended in the appropriate K^+ solutions just before use. This insured constant internal K^+ in all the samples and helped to set external K^+ to precise values.

Rationale

The method consists simply of following changes in pH in an unbuffered external medium with a conventional glass electrode. When H^+ is in equilibrium, the membrane potential, V_m, will be given by:

$$V_m = \frac{RT}{F} \ln \frac{a_o}{a_i} = 2.303 \frac{RT}{F} \cdot (pH_i - pH_o) \tag{1}$$

where a_o, pH_o and a_i, pH_i are the H^+ activities and pH on the outer and inner sides of the cell membrane and RT/F is the gas constant times absolute temperature divided by the Faraday constant. On the other hand, the potential, V_H, measured by a glass electrode in the external medium is given by:

$$V_H = -2.303 \frac{RT}{F} \cdot pH_o + V_H^0 \tag{2}$$

where V_H^0 is a constant (independent of pH_o). Eliminating pH_o from Eqns. 1 and 2, we have:

$$V_m = V_H + 2.303 \frac{RT}{F} \cdot pH_i - V_H^0 \tag{3}$$

If V_m changes by an amount ΔV_m, the corresponding changes in V_H and pH_i will be related by:

$$\Delta V_m = \Delta V_H + 2.303 \frac{RT}{F} \cdot \Delta pH_i \tag{4}$$

If the intracellular buffer capacity is much larger than the buffer capacity of the suspension medium, then changes in the internal pH can be ignored. Setting $\Delta pH_i = 0$ in Eqn. 4, we see that changes in membrane potential are identical to changes in the potential recorded by a glass electrode. For absolute values, the method only requires a single calibration point which can always be obtained from measurements of the Cl^- ratio when the cells are in a steady state.

To validate the method we shall show that: (1) experimental conditions are easily arranged so that pH_i remains constant when the potential changes, (2) low concentrations of H^+ carriers cause a rapid H^+ equilibration, and (3) the measured membrane potential responds in the anticipated way to changes in both anion and cation composition of the media.

Drugs and chemicals. Sources of drugs used in these experiments were: nystatin and carbonyl cyanide *m*-chlorophenyl hydrazone (CCCP) from Sigma Chemical Co. (St. Louis, Mo.); 3,3',4',5-tetrachlorosalicylanilide (TCS) from Eastman Organic Chemicals (Rochester, N.Y.); valinomycin from Calbiochem (San Diego, Calif.); phloretin from K and K Chemical Co. (Plainview, N.J.); 4-acetamido-4'-isothiocyano-2,2'-disulfonic stilbene (SITS) from Polysciences (Warrington, Pa.); 4,4'-diisothiocyano-2,2'-disulfonic stilbene (DIDS) from Pierce Chemical Co. (Rockford, Ill.); antibiotic A23187 a gift of Dr. Robert L. Hemill of Eli Lilly and Co. (Indianapolis, Ind.). TCS, CCCP, valinomycin, nystatin and phloretin were administered to the cells as concentrated solutions in ethanol. A23187 was dissolved in 2 : 1 (v/v) ethanol/dimethylformamide. SITS was applied as an aqueous solution and DIDS was added as a powder to the cells being treated.

Electrode measurements. Glass K^+ and pH electrodes from Microelectrodes, Inc. (Londonderry, N.H.) were used for ion measurements. The low Na^+/K^+ selectivity of the K^+ electrodes (about 5/1) necessitated choline chloride backgrounds for experiments in which K^+ was measured. K^+-selective electrodes based upon valinomycin dissolved in a plastic matrix [11] were also tried, but proved unsuitable in experiments involving the hydrogen carriers (TCS and CCCP) due to interference with electrode function. The valinomycin-based electrodes were used to measure internal K^+ of the cells, however, because of the presence of Na^+. Their very high K^+/Na^+ selectivities (about 5000/1) reduced the errors in these measurements.

Other procedures. The internal pH was estimated by sonicating packed cells. A glass electrode was used to measure the pH of the disrupted suspension. Internal K^+ was similarly measured in sonicated cells using K^+ electrodes.

Results

Constant internal pH and the effect of hematocrit

From Eqn. 4 one may see that a condition for accurate measurement of membrane potential changes is that the internal pH change be very small. The buffering capacity of the cells should insure this condition. For example, when the cells are hyperpolarized (i.e. the inside becomes more negative) the pH of the external solution becomes more alkaline. If the cells are in an unbuffered medium originally at pH 7, this will be accomplished by the passage of a very small number of H^+ into the cell (or OH^- out of the cell) which can

easily be accommodated by the intracellular buffers.

The actual magnitude of the change in internal pH in any given situation can be estimated as follows. Let β_o and β_i denote the buffer capacities of the solutions on the outer and inner sides of the membrane. Then by definition, the change in pH which results from the addition of ΔH mol of H^+ will be:

$$\Delta pH_o = \frac{\Delta H_o}{\beta_o} \qquad \Delta pH_i = \frac{\Delta H_i}{\beta_i} \tag{5}$$

But any addition (or subtraction) of H^+ in the inner compartment will be at the expense of the outer compartment. Thus, if v_i and v_o are the water volumes of the inner and outer compartments:

$$v_o \Delta H_o = -v_i \Delta H_i \tag{6}$$

Eliminating ΔH_i and ΔH_o from Eqns. 5 and 6 leaves:

$$\Delta pH_i = \frac{\beta_o v_o}{\beta_i v_i} \Delta pH_o \tag{7}$$

Inserting Eqn. 7 into Eqn. 4 and using Eqn. 2 to calculate ΔpH_o we have:

$$\Delta V_m = \Delta V_H(1 + \epsilon) \tag{8}$$

where:

$$\epsilon = \frac{\beta_o v_o}{\beta_i v_i} \tag{9}$$

represents the error in the estimation of ΔV_m by external pH measurements.

To utilize Eqn. 8, note that β_i = 62 mM [11]. Further, assuming that approx. 72% of the cell volume is available for dissolved solutes, the ratio v_o/v_i is related to the hematocrit, h, by $v_o/v_i = (1 - h)/0.72h$. Inserting these values into Eqn. 8 yields:

$$\epsilon = 0.022\beta_o(1 - h)/h \tag{10}$$

Note that $\epsilon = 0$ only when either $\beta_o = 0$ or $h = 1$. In practice, neither of these conditions will ever be fulfilled. However, β_o for an unbuffered solution of NaCl (pH 7) will be equal to 10^{-4} mM so that ϵ will be negligible for any reasonable value of h. If the solution is equilibrated with atmospheric CO_2 (pCO_2 = 0.3 mm Hg), then β_o has a maximum value of $8 \cdot 10^{-3}$ mM and ϵ will be less than 0.01 whenever h is greater than 1.7%. Finally, if we accept $\epsilon = 0.01$ as the maximum tolerable error, then it can be seen from Eqn. 9 that this can always be accomplished by choosing: $h > 2.2\beta_o$, where β_o is in mM.

These considerations are verifed by the data in Table I. Potassium permeability was increased by adding ionophore A23187 to cells in media containing Ca^{2+} (Gardos effect) [12,13]. By adjusting the external K^+ concentration the membrane potential was changed over an 80 mV range and the internal pH of sonicated cells was measured. The internal pH values were constant, with maximum variation of ±0.025 pH (corresponding to a maximum variation in V_m of ±1.5 mV). These data show that in the absence of extracellular buffer, the internal pH is independent of membrane potential.

Eqn. 9 predicts that the method can be used at quite low hematocrits pro-

TABLE I

CONSTANCY OF INTERNAL pH AS THE MEMBRANE POTENTIAL IS VARIED

Red blood cells were suspended in NaCl/KCl mixtures (total concentration = 150 mM) to vary the membrane potential when K^+ permeability was increased by addition of A23187 (1 μM) plus Ca^{2+} (0.5 mM). The jump in potential (V_H) when A23187 was added was recorded. Cells were treated with TCS (4 μM) to accelerate H^+ equilibration.

YVH

ΔV_H (mV)	pH of sonicated cells	ΔV_H (mV)	pH of sonicated cells
8.0	7.32	−15.2	7.30
7.0	7.31	−20.0	7.31
4.0	7.30	−22.8	7.29
2.0	7.29	−24.0	7.29
1.4	7.32	−31.5	7.29
1.0	7.32	−33.0	7.27
0	7.30	−35.6	7.27
−2.0	7.30	−40.0	7.27
−5.0	7.30	−54.8	7.29
−6.6	7.29	−59.4	7.30
−10.0	7.32	−71.8	7.28
−13.7	7.31		

vided that external buffering is minimized. Table II shows that this is true. Cells were suspended in 150 mM KCl and adjusted to different hematocrits. To each aliquot of cells a volume of 260 mM sucrose equal to the external volume was added to reduce ion concentrations to one half, and V_H was measured. There was no significant difference in ΔV_H when the final hematocrit varied from 28 to 2.6%. To achieve results such as these it is important that the cells be properly washed and that no hemolysis takes place, releasing intracellular buffer. Voltage deviations at hematocrits as high as 7% have been observed when these precautions were not followed.

Speed of response

The response time of our system, illustrated in Fig. 3 was measured by

TABLE II

INDEPENDENCE OF V_H AND HEMATOCRIT

Cells were suspended in 150 mM KCl plus 0.5 mM $CaCl_2$. External Cl was diluted 2-fold by addition of 260 mM sucrose and ΔV_H was observed.

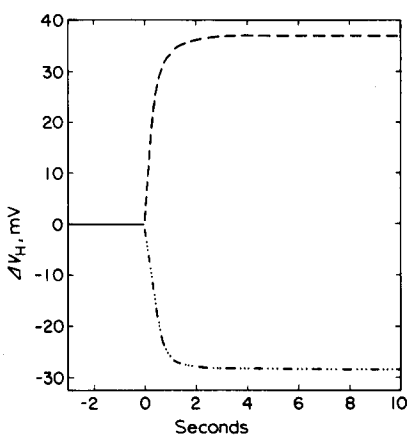
Initial hematocrit (%)	Final hematocrit (%)	ΔV_H (mV)
44.0	28.2	15.0
35.0	21.2	15.1
23.7	13.4	15.4
15.6	8.5	15.3
12.5	6.7	14.1
8.1	4.2	15.3
5.0	2.6	15.5

adding small amounts of acid or base to the cuvette and measuring the voltage output of the glass electrode. The speed of response is determined largely by the efficiency of mixing and the response time of the electrode. Fig. 1 shows that the system response is 95% complete by 1 s.

The response time of V_H was tested in cells treated with valinomycin to raise K^+ permeability and with DIDS to reduce Cl^- permeability. Fig. 2 illustrates the ΔV_H resulting from the sudden dilution of external K^+. In such cells, the K^+ permeability dominates and V_m is driven toward the K^+ equilibrium potential. The change in V_H is in the anticipated direction, but the response is very sluggish (uppermost curve of Fig. 2). However, addition of low doses of H^+ carriers (CCCP in this instance) markedly accelerate the rate of equilibration. In this experiment maximum acceleration took place at and above a CCCP concentration of 20 micromolar. TCS was as effective as CCCP, but two other carriers, pentachlorophenol and dinitrophenol required much higher doses to achieve the same results.

Although H^+ carriers by themselves have little or no effect upon K^+ permeability, they act synergistically to increase both the valinomycin- and the Ca^{2+}-mediated permeability changes. This is shown by increased K^+ efflux, a faster rate of shrinking and by a greater hyperpolarization in the presence of H^+ carriers. These effects which may be due to K^+-H^+ exchange [14] are currently under investigation.

In contrast to the results with valinomycin, depolarization or hyperpolarization of the membrane by anion substitution (e.g. benzoate or isethionate for Cl^-) leads to a very rapid response. In this case, addition of H^+ carriers does not produce any detectable change in the magnitude or speed of response (Fig. 3).



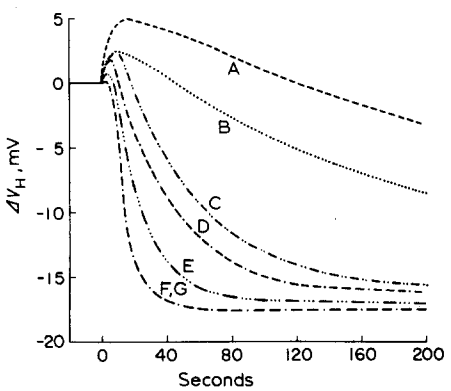


Fig. 1. Response time of pH electrodes. Electrode was immersed in 150 nM NaCl buffered to pH 7.4 with 5 mM HEPES. At zero time HCl (upper curve) or NaOH (lower curve) was added to the solution.

Fig. 2. Use of a hydrogen ionophore (CCCP) to speed the H^+ equilibration in red blood cell membranes. Red blood cells were treated with DIDS (1 mg/cm^3 packed cells) to lower chloride permeability and with valinomycin (0.1 μmol/cm^3 packed cells) to raise potassium permeability. The cells were suspended in 100 mM KCl plus 40 mM NaCl at a 20% hematocrit. At time zero the external K^+ concentration was diluted 2.25 times by the addition of 150 mM NaCl. The following concentrations of CCCP were used: curve A, 0; curve B, 0.2 μM; curve C, 0.5 μM; curve D, 1 μM; curve E, 2 μM; curve F, 20 μM; curve G, 50 μM.

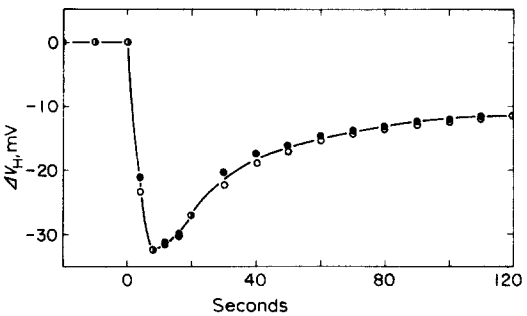


Fig. 3. Hydrogen ionophore (CCCP) does not affect H^+ equilibration when one anion is substituted for another. Red blood cells were suspended in 150 mM NaCl at a 16.4% hematocrit. At zero time 150 mM sodium benzoate was added to a final concentration of 75 mM. CCCP concentration was 20 μM. •, controls; ○, CCCP treated.

Evidence that the rapid pH response involves the anion exchanger is presented in Fig. 4 where the response is slowed considerably in the presence of 2 mM phloretin, a potent inhibitor of anion exchange [15,16].

Changes in V_m with changes in anion concentration

Normally, Cl^- is equilibrated across the red cell membrane, and V_m equals the Cl^- equilibrium potential. It follows that if external Cl^- is progressively diluted with an impermeant anion, then a plot of V_m against $\log[Cl_o^-]$ will yield a straight line with a slope of about 60 mV. The results illustrated in Fig. 5, curve 1 show that when Cl^- is replaced with isethionate a straight line is approached only at the highest Cl^- concentrations. Presumably isethionate is not entirely impermeable (a more detailed analysis indicates that the permeability ratio Cl^-/isethionate is about 3). When Cl^- both inside and outside the cell is replaced with the more permeable benzoate ion, and ΔV_m is measured in various mixtures of benzoate and isethionate, curve 2 of Fig. 5 is obtained.

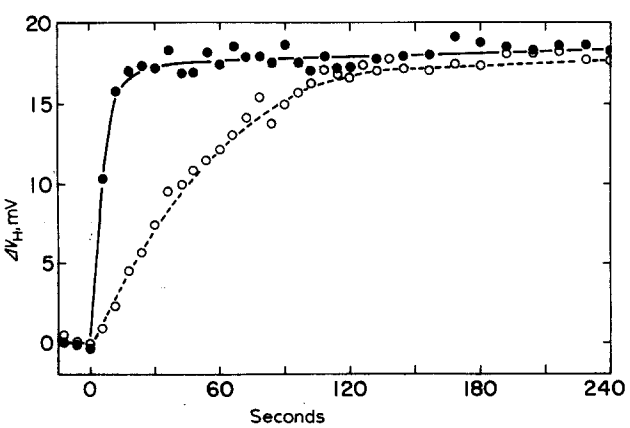


Fig. 4. Phloretin inhibition of H^+ equilibration. Cells were suspended in 150 mM NaCl at a 30% hematocrit. At zero time 150 mM sodium isethionate was added to a final concentration of 88 mM. Phloretin concentration was 4 mM. •, control, ○, phloretin treated.

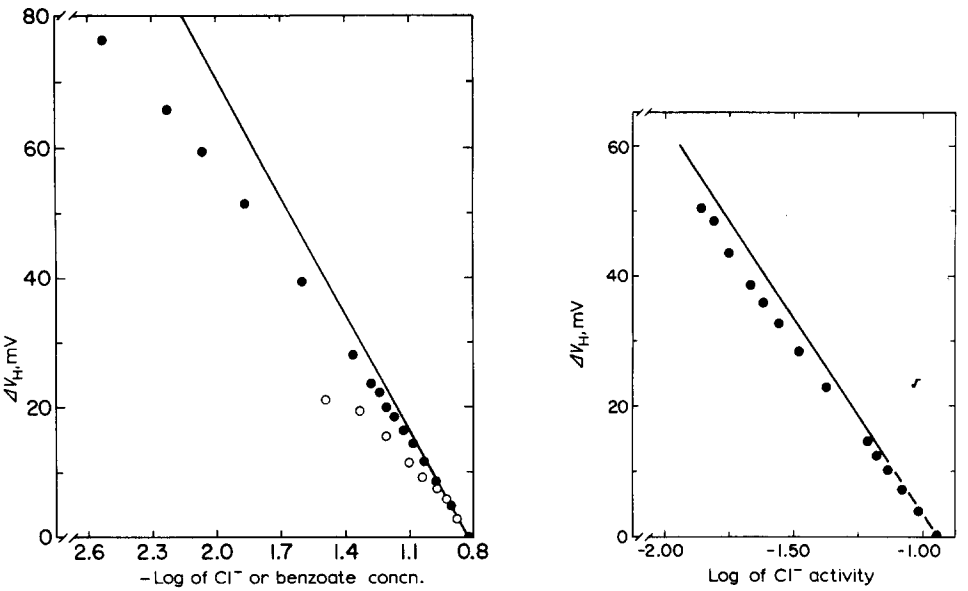


Fig. 5. Approach to the theoretical Nernst slope in anion substitution experiments. The solid line has the theoretical slope of 60.15 mV. The bottom curve (○) shows an experiment in which the cells were originally suspended in 150 mM NaCl. Partial replacement of chloride with isethionate yielded the curve shown. For the experiment shown in the upper curve the internal Cl^- was replaced with benzoate by repeated incubation. The cells were then suspended in 150 mM sodium benzoate. Partial replacement of the benzoate with isethionate gave a curve (●) approaching the theoretical much more closely.

Fig. 6. Change in V_H when external Cl^- is diluted with isotonic sucrose. The solid line indicates the theoretical slope. Cells were suspended in 150 mM KCl (hematocrit = 46%) and dilutions were made with 270 mM sucrose.

The slope of this line approaches 60 mV over a much wider concentration range than the corresponding slope in Cl^- solutions. This is the anticipated result because benzoate is much more permeable than Cl^- and is, therefore, more effective in swamping out the effects of isethionate. Finally, the theoretical equilibrium potential slope can be very closely approximated if normal cells in an isotonic NaCl solution are progressively diluted with isotonic sucrose. The results shown in Fig. 6 are completely in accord with expectations.

Measurements of membrane potential during anion substitution give important information about both the conductive (leakage) and the exchange pathway for anion transport. Thus, when external Cl^- is diluted by anion substitution two patterns of voltage response are seen. The first of these is illustrated by isethionate (Fig. 4, upper curve). In this case ΔV_m is positive, which is what one would expect for anions less permeable than Cl^-. For these anions, the voltage, after reaching its peak, drops very slowly towards the starting point. The second pattern is typified by benzoate (Fig. 3). For this class of anions the voltage change is negative, indicating a higher permeability than Cl^-, and the decay of the potential is very rapid. In each case, the peak of the jump in membrane potential can be used to estimate the relative conductive permeabilities of Cl^- and the substituted anion.

The decay of the membrane potential appears to be caused by changes in

anion concentrations as Cl^- moves out of the cell and is replaced by the probe anion. This process takes place chiefly through the electrically neutral exchange-diffusion system which is 10^4 times faster than the conductive pathway [17—19]. Thus, the entire time course of the exchange process can be simply obtained from the decay of the potential. For this class of anions the voltage change is negative, indicating a higher permeability than Cl^-, and the decay of the potential is very rapid.

Changes in V_m with changes in cation permeability

Since the "electrogenic" permeability of Cl^- is some 100 times greater than cation permeabilities it follows that V_m will be closely approximated by the Cl^- equilibrium potential. However, there are a number of experimental circumstances where the K^+ permeability is increased by orders of magnitude [20]. If the K^+ permeability becomes sufficiently larger than the Cl^- permeability, then V_m will no longer depend on Cl^-, but will become approximated by the K^+ equilibrium potential.

Potassium permeability can be elevated by the addition of small amounts of Ca^{2+} together with the divalent cation ionophore A23187. The ionophore carries external Ca^{2+} into the cell where it induces a specific K^+ leakage [12,13], the "Gardos effect". Fig. 7 shows the results of one such experiment where ΔV_m is plotted against $\log[K_o^+]$. The experimental points (open circles) fall far short of the theoretical K^+ equilibrium potential. Presumably, the increased K^+ permeability is not large enough to swamp the Cl^- permeability. One would expect the slope to improve if the Cl^- permeability was inhibited at the same

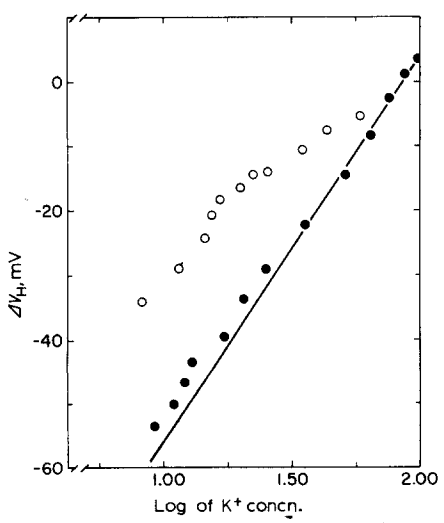


Fig. 7. Approach to the Nernst slope in red blood cells with high potassium permeability. Cells were suspended in choline chloride/KCl mixtures (total concentration = 150 mM) plus 0.5 mM $CaCl_2$ at a hematocrit of 30%. All cells were treated with CCCP (20 μM) to speed H^+ equilibration. A23187 (2 μM) was added to increase K^+ permeability via the Gardos effect. Voltage jumps upon addition of A23187 varied with K^+ concentration as shown. The solid line indicates the theoretical slope for a perfect cation-selective membrane. One group of cells (•) was treated with DIDS (1 mg/3 cm^3 packed cells) to reduce Cl^- permeability and further increase the K^+/Cl^- permeability ratio.

time that the K^+ permeability was increased. This was accomplished by inducing the Gardos effect in cells treated with the anion transport inhibitor DIDS [21,22]. The results are illustrated in curve 2 of Fig. 7, which shows that the slope now very closely approaches the theoretical K^+ equilibrium potential.

Discussion

The very rapid equilibration of H^+ which follows anion substitution probably involves the anion-exchange system. When the relatively impermeable isethionate is substituted for Cl^- in the external medium, there will be a rapid Cl^-—OH^- (or Cl^-—HCO_3^-) [23] exchange until the exchanger comes into equilibrium, with $Cl_i^-/Cl_o^- = OH_i^-/OH_o^-$. At this point the corresponding equilibrium potential will be set up by anion diffusion through the electrogenic path. The equilibration process is rapid because mass movements take place primarily through the fast exchanger, while establishment of the equilibrium potential through the sluggish conductive pathway is rapid because very few ions are required to charge up the membrane.

On the other hand, if the membrane potential is altered by increasing K^+ permeability, H^+ equilibration in the absence of ionophore is slow. In this case there is no initial drive for the anion exchanger since the concentration ratios of Cl^- and OH^- are still equal eventhough this ratio is not in equilibrium with the membrane potential. The only recourse is for ions to diffuse through the slow electrogenic path. Of course, the anion exchanger will become active the moment the concentration ratios move off of equilibrium, but the fact remains that the entire process will be rate limited by diffusion through the slower electrogenic paths.

Although the pH changes which occur during ion movements in red blood cells have been noted by several investigators [23,10], previous studies have focused upon kinetics and stoichiometry of the phenomenon. We have tried to establish the conditions under which such pH changes accurately reflect changes in the membrane potential, so that external pH measurements can be used to monitor ΔV_m. The chief advantage for using H^+ instead of Cl^- distribution for estimating membrane potentials is that it is not restricted to the steady state. In addition, rapid and continuous measurements of H^+ are much simpler than the corresponding measurements of Cl^-. Further, because of the constant internal pH, intracellular measurements are not required for estimates of change in membrane potential. For absolute values, the method only requires a single calibration point which can always be obtained from measurements of the Cl^- ratio when the cells are in a steady state.

The principle advantage over the fluorescent dye method is that the H^+ distribution method is simpler both in theory and practice. The fluorescent dye method is based on the observation that the amount of cationic dye associated with the cells increases with membrane hyperpolarization. The distribution of dye between cell and medium can be detected because emission from cell-associated dye becomes quenched, presumably because of formation of dye aggregates which have reduced fluorescence [24]. Since the mechanism of dye uptake as well as aggregate formation is not established [24], the linear correlation that is found in red cells between membrane potential and fluorescence

remains a fortuitous empirical result. It follows that careful independent calibrations are required under different sets of experimental conditions. For examples, the fluorescent signal intensity is different in high K^+ and low K^+ cells, a number of transport inhibitors interfere with the fluorescence, emitted light scattering is dependent on cell volume, and the fluorescence-membrane potential relationship is non-linear in squid axons and in Ehrlich ascites tumor cells [4,25].

In contrast, the H^+ distribution is based upon an elementary thermodynamic argument which allows the method to be applied to a wide variety of situations. There is no problem with using either concentrated or dilute (down to at least 3% hematocrit) cell suspensions. No calibration is necessary for measurements of changes in potential, and when absolute values are required, calibration is relatively simple. Of course, the method fails completely when non-equilibrium H^+ distributions are under study (e.g. in mitochondria).

The method is especially attractive in determining relative anion permeabilities and other anion transport parameters. In contrast to other methods for studying anion transport, the conductive and exchange-diffusion pathways are relatively easy to separate and measurements of both may be obtained in the same experiment.

At first sight, the unorthodox procedure of leaving the medium unbuffered seems to be a disadvantage. However, in any system like red cells, where H^+ equilibrate, any change in potential will be necessarily followed by a change in pH gradient across the membrane. If the external medium is heavily buffered (say by a pH stat), then the only recourse will be for the internal pH to change. Omitting the external buffer means that the internal pH remains constant and all the pH change occurs in the external medium where at least it is measurable.

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