J. Membrane Biol. 12, 69–88 (1973)

A Study of Passive Potassium Efflux from Human Red Blood Cells Using Ion-Specific Electrodes

F. M. Morel

Department of Engineering, California Institute of Technology
Pasadena, California 91109

Received 19 May 1972

Summary. Using ion-specific electrodes, the potassium leakage induced by ouabain in human erythrocytes can be measured continuously and precisely near physiological conditions. Upon small additions of isotonic sucrose solution to a suspension of red cells in physiological saline the passive potassium efflux increases proportionally to the chloride ratio. The same result is obtained upon addition of hypertonic sucrose solution, suggesting that neither osmolarity nor intracellular concentrations have any influence on the passive potassium efflux. The independence of the potassium efflux and osmolarity can be verified by addition of a penetrating substance like glucose to the cell suspension. Adding water or hypertonic sodium chloride solution shows that the potassium efflux increases slightly in more concentrated salt solutions. Inasmuch as it can be interpreted as a pure ionic strength effect, this result supports the hypothesis of independence of potassium efflux and intracellular concentrations. The results of this investigation together with other studies show that the passive permeability of the human red blood cell to potassium depends uniquely on the membrane potential near physiological conditions, while it depends on parameters such as pH or concentrations for large membrane potentials. This suggests that two different mechanisms of transport might be involved: one would control the permeability under normal conditions; the other would represent a leak through the route normally used by anions and become important only under extreme conditions.

Because it is readily available in great quantities and its structure is simpler than that of other cells, the erythrocyte is an object of choice for the study of cell membrane properties. The active pumping of sodium and potassium across the red cell membrane has been demonstrated for some time [13] and it is probably now the best studied of all active transport processes.

A necessary lemma to the complete elucidation of active transport mechanisms in the red cell membrane is the explanation of the cell's passive transport properties. How does the red cell discriminate between ions and, above all, between anions and cations? What parameters determine the

flux of each ion? How do the ionic fluxes depend on those parameters? Such questions must be answered if one is to explain the ionic steady state across the red cell membrane.

The most striking fact concerning the passive permeability of the red blood cell membrane to ions is their ability to discriminate between anions and cations. For example, it has been shown that chloride crosses the membrane about a million times faster than potassium [23]. Such behavior is the main basis for the development of the fixed-charge hypothesis which attempts to explain the permeability characteristics of the erythrocyte membrane by supposing the existence of fixed cations in the aqueous channels within the membrane. In view of the demonstrated negative charge on the surfaces of the cells [25], the fixed cations are thought to be located inside the membrane, somewhere in the pores through which the hydrophilic ions are supposed to migrate. The model is then that of an ion exchanger membrane. The behavior of such membranes has been extensively studied [7] theoretically and experimentally, and predictions can be made on which to test the model for the erythrocyte membrane.

One of the most direct supports of the fixed-charge hypothesis for human erythrocytes comes from studies with specific reagents. For example, 1-fluoro-2,4-dinitrobenzene which reacts strongly with amino groups has been shown to increase cationic fluxes while decreasing anionic fluxes [16].

Detailed studies of the penetration of polyvalent anions in red cells [3, 10, 12, 15, 16] have shown that the fluxes are uniquely functions of an intramembrane concentration computed on the basis of the fixed-charge hypothesis. Even though the fluxes depend exponentially instead of linearly on this intramembrane concentration, Passow considers these results a strong support for the fixed-charge hypothesis.

An attempt to obtain support for the fixed-charge hypothesis by measuring cationic fluxes was made by Wilbrandt and Schatzmann [26] and then LaCelle, Rothstein and Donlon [5, 11, 20]. By suspending red blood cells in low ionic strength media whose isotonicity was maintained by sucrose, they found that the curve "rate of salt leakage" *vs.* "logarithm of outside ionic concentration" is made up of three linear segments with markedly increasing slopes as the ionic concentration decreases. The authors interpreted their results using Goldman's constant field equation for the flux of ions

$$J_\alpha = -P_\alpha \frac{[\alpha]_i \exp(-z_\alpha V_m) - [\alpha]_o}{\exp(-z_\alpha V_m) - 1} V_m \tag{1}$$

where J_α is the flux of the ion α; P_α is a permeability coefficient; z_α is the charge of the ion; $[\alpha]_i$ and $[\alpha]_o$ are the ion activities on each side of the

membrane; V_m is the adimensional electrical potential across the membrane: $V_m = \frac{\psi F}{RT}$ (ψ = membrane potential; F = Faraday constant; R = gas constant; T = absolute temperature).

The difference of potential V_m is given by the Donnan ratio of the chloride ion:

$$V_m = \ln \frac{[Cl]_i}{[Cl]_o}. \tag{2}$$

The results were then explained by the application of Eq. (1) and a permeability coefficient could be computed for each linear segment.

Barr [1] has shown that the Goldman equation for the diffusion potential across a membrane

$$V_m = \ln \frac{\sum P_- [\alpha_-]_i + \sum P_+ [\alpha_+]_o}{\sum P_- [\alpha_-]_o + \sum P_+ [\alpha_+]_i} \tag{3}$$

is valid in a number of cases where the field is far from constant. Eq. (3) reduces to Eq. (2) if Cl is the only anion and if P_{Cl} is much larger than the permeability coefficients of all the cations. (The permeability coefficients P_α used here are directly proportional to Goldman's "mobilities.") However, this does not justify the use of Eq. (1) for the fluxes of ions unless, as has been shown by Teorell [22], the total concentrations of diffusible species are equal on both sides of the membrane. This is clearly not the case in experiments where cells are suspended in low ionic strength media. Furthermore, the difference of potential V_m which appears in the expression of the fluxes of ions across an ion exchanger membrane is the diffusion potential across the membrane and bears little relationship to the total membrane potential which is the sum of V_m and of the potentials at the inner and outer surfaces of the membrane. To obtain the diffusion potential V_m, Eq. (3) should be applied to the concentrations at the limits of the membrane, not in the bulk.

As noted by Passow [16] Wilbrandt's, Rothstein's and others' results cannot then be taken as a verification of the fixed-charge hypothesis. In fact, they demonstrate an unexpected and unique influence of the total electrical potential across the erythrocyte membrane on the cationic fluxes. This phenomenon was further documented in a recent paper by Cotterrell and Whittam [2] who studied the effect of substituting EDTA or citrate for chloride in the extracellular medium. They found little effect on active (ouabain-sensitive) cationic fluxes whereas the passive (ouabain-insensitive) fluxes of both sodium and potassium were markedly affected by an increase in the membrane potential. The authors concluded that the membrane

potential affects the permeability of the red cell membrane in an asymmetric way with respect to influxes and outfluxes of cations. This explanation is not really satisfactory as it does not consider the possibility of changes in driving forces.

Further experiments are warranted to clarify the nature of the total membrane potential influence on the cationic fluxes. This is one of the questions that the present investigation will attempt to answer: How do the passive cationic fluxes depend on the concentrations and the total membrane potential?

Two principal methods have been used to study cationic transport across red cell membranes: radioactive tracers (e.g., Cotterrell & Whittam [2]) and conductivity measurements (e.g., LaCelle & Rothstein [11]). One of the purposes of this investigation is to test the feasibility of a new technique to follow net ionic fluxes through the red cell membrane. Using ion specific electrodes to measure the activities of various ions in the extracellular medium, it should be possible to monitor continuously the corresponding net ionic fluxes through the membrane. We shall see that the technique is mainly useful to measure potassium fluxes.

One has direct access to the extracellular medium in order to vary the experimental conditions. An indirect access to the intracellular medium is obtained through the osmotic properties of the red cells: intracellular concentrations can be increased or decreased by changing osmotically the red cell volume. These means of varying intracellular and extracellular concentrations will be used to study how the potassium efflux depends on parameters such as intracellular potassium concentration and membrane potential principally. As we want to limit this investigation to the study of passive fluxes, the red cells will be poisoned with ouabain, which has been shown to block specifically the active transport mechanisms [19].

By increasing our knowledge of the detailed phenomenological aspects of the passive transport of potassium across the red cell membrane, we hope to throw some light on the transport mechanism itself.

Materials and Methods

General Set-Up

A red blood cell suspension is kept in the inner chamber of a 250-ml jacketed Pyrex beaker. Water from a constant temperature bath (Radiometer VTS · 13c) circulates in the jacket. The temperature of the system is then maintained at 37 ± 0.1 °C and it is measured by a thermocouple (Yellow Springs Instruments 43 TA). A magnetic stirrer keeps the suspension well mixed. The electrodes are mounted on the cover of the beaker

and dipped directly into the cell suspension. They are connected to a switching box (Orion-605) and their output is read on a digital pH-mV meter (Orion-801). A constant voltage regulator is interposed between the meter and the electrical outlet to insure maximum stability in the readings. Because of the high impedance of some of the electrodes, it was necessary to enclose the system in a Faraday cage.

Electrodes

Eight electrodes are usually used simultaneously: two reference, two potassium, one chloride, one pH, and two sodium electrodes.

Either of the two reference electrodes can be used in conjunction with any of the other six electrodes. One is a single fiber junction calomel electrode (Beckman 39410) filled with saturated KCl; the other one is a sleeve-type Ag–AgCl electrode (Orion 90-01) filled with a special solution (Orion 90-00-01). It was found that in red blood cell suspensions, the potassium leakage from the reference electrodes is always negligible, probably as a result of partial clogging of the liquid junctions. This will be seen in the first part of Fig. 3: before the cells are added in the saline solution, the potassium readings are stable within the accuracy of the method. The use of the two electrodes simultaneously allows a better control of the drift they sometimes undergo.

The chloride ion electrode is a liquid ion exchanger electrode (Orion 92-17).

The sodium and the pH electrodes are glass electrodes (sodium: Corning 476210 and Beckman 39278; pH: Beckman 39301).

The two potassium ion electrodes are identical liquid ion exchanger electrodes (Orion 92-19). The liquid ion exchanger is a solution of valinomycin absorbed on a porous membrane. The internal filling solution is usually 0.1 M KCl. These potassium electrodes were found to age rather rapidly (a few days to a few weeks). They, then, have a reduced response slope and tend to give erratic readings. This is the reason why two of them are used simultaneously: one is used as a back up for the other.

A high selectivity for potassium over sodium of these electrodes is the main characteristic enabling one to measure small potassium concentration increments in a solution containing principally sodium chloride. This selectivity was found to be as high as the manufacturer claimed: about 10^4. A typical calibration curve for a new electrode is

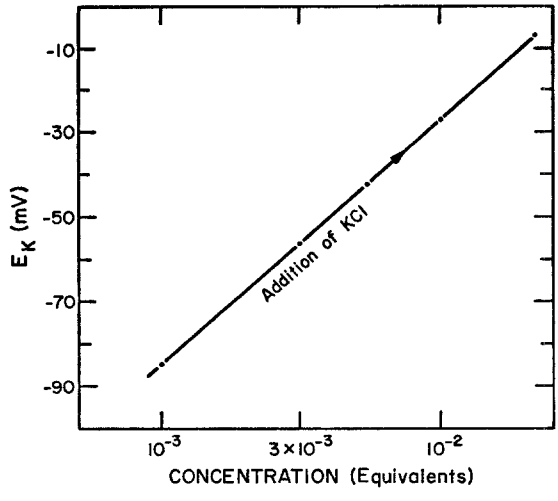

Fig. 1. Calibration curve of the potassium electrode in isotonic buffered saline

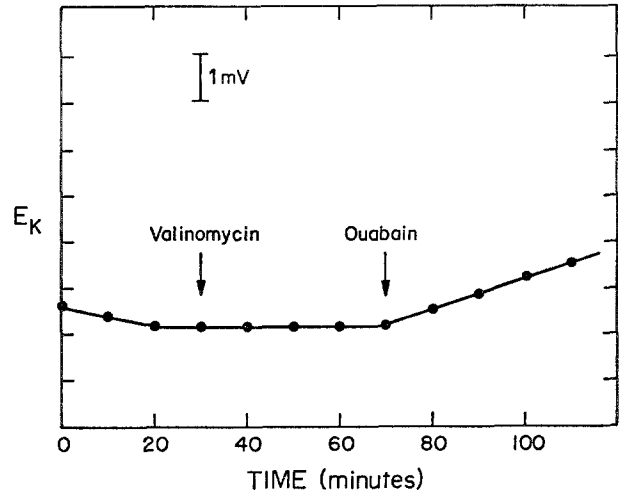

Fig. 2. Addition of 0.5 ml of potassium ion exchange solution (valinomycin) to 70 ml of a 31% red blood cell suspension in physiological saline. Subsequent addition of ouabain produces the usual increase in the readings (*see* Results)

shown in Fig. 1. Note that the calibration was performed with an excess of sodium ions and that the slope is near the theoretical one (59 mV instead of 61.5 mV). Although the electrodes demonstrated an unexplained hysteresis upon addition of water in the measured solution [14] this was not considered important as only increases in potassium concentration were actually measured. It was verified by the method of known additions that the calibrations in saline were applicable to cell suspensions. It was also verified that the electrodes were insensitive to the presence of glucose or sucrose in the solution.

The presence of the valinomycin solution in the porous membrane of the potassium electrode could introduce a major artifact. The valinomycin could slowly dissolve into the aqueous phase and, from there, into the erythrocyte lipids. The potassium outflux would then be increased markedly as has been shown by Tosteson, Cook, Andreoli and Tieffenberg [24]. To verify that this effect is either too small or too slow to affect the measurements significantly, an experiment was performed where 0.5 ml of the valinomycin solution was added directly to the cell suspension. The organic solution was seen to be immiscible with the aqueous one and to float on top of it. Although the surface of contact between the two phases was increased by a factor of about 100 (from a few mm^2 in the membrane of the potassium electrode to some cm^2 on top of the suspension), thus facilitating the transfer of the valinomycin, it did not affect the red blood cells in any measureable way. This can be seen in Fig. 2. The failure to measure any effect was not due to some electrode defect, as addition of ouabain resulted in the usual increase in the slope of the potassium readings. (*See* Results.)

Osmolarity and Haematocrit

The osmolarity and the haematocrit of the cell suspension were measured when needed by withdrawal of 0.6-ml samples. About 0.2 ml were used for osmotic measurements with a freezing point depression osmometer (Osmette 2007-Precision Systems); the rest, sucked into haematocrit capillaries, was spun for 6 min at 13,000 $\times g$ in a micro-

capillary centrifuge (International Equipment Co. — Model MB). The packed cell volume was then read on an optical comparator (International Equipment Co. — Model CR). By taking the solid (nonaqueous) volume of the cells to be 30% of their total packed volume under isotonic conditions [18], the intracellular aqueous volume could be computed at all times.

Red Blood Cell Suspensions

O^+ type blood from healthy donors was obtained from the blood bank in standard 500 cc bags with 75 cc of acid citrate dextrose solution (ACD) as anticoagulant. For each experiment, 80 cc samples were incubated for three hours at 37 °C with about 40 mg of adenosine added to their plasma. The samples were then spun down at $3{,}000 \times g$ for 10 min. The plasma and the buffy coat were removed and the cells washed one time in a buffered saline solution (4.40 g NaCl, 0.68 g KH_2PO_4, 0.69 g $NaH_2PO_4 \cdot H_2O$, 15.2 g $Na_2HPO_4 \cdot 7\,H_2O$ in 1 liter of water). This preparation yielded about 30 cc of washed packed red cells that were added to 40 cc of the saline solution in which the electrodes were allowed to equilibrate. The pH of this suspension was usually very close to 7.0 and the isotonic haematocrit around 40%. Ouabain (Strophanthin G-Calbiochem) was usually added later to the cell suspension at a concentration of approximately 10^{-4} M.

Correction for Drift; Computation of Ionic Fluxes and Precision

The concentrations present in the extracellular saline solution at the start of an experiment were the following: $(K^+) \simeq 5$ mEquiv; $(Na^+) \simeq 195$ mEquiv; $(Cl^-) \simeq 75$ mEquiv; (phosphates) $\simeq 67$ mEquiv[1]. For a typical K^+, Na^+ exchange of 1 mEquiv per liter of red blood cells, per hr, the actual changes in concentration should be of about $1 \times \frac{40}{60} \approx 0.67$ mEquiv/liter of saline per hr. This is a 13% increase in potassium concentration and a 0.3% decrease in sodium concentration per hr. For ideally-behaving electrodes this would lead to about $+3.1$ mV and -0.07 mV changes per hr, respectively. Given a precision in the readings of ± 0.1 mV, it can be seen that in the same one-hr-long experiment, the outflux of potassium can be easily measured, while an equal influx of sodium cannot. This great insensibility in the sodium measurements allows one to correct for drifts in the reference electrodes. Any drift that exceeds markedly the expected changes in the sodium ion electrode readings can be corrected for. The presence of two such electrodes allows one to verify that the drift is indeed due to the reference electrode and not to the sodium electrodes themselves.

A further refinement in this correction is given by the comparison of the sodium electrodes with the chloride one. As the slope of the chloride ion electrode is negative (the readings in millivolts decrease as the chloride concentration is increased) while all the others are positive, any similar variations in the sodium and chloride electrodes' responses have to be due to variations in the common reference electrode. Otherwise, these variations would mean either similar identical drifts in independent electrodes or a similar relative increase in sodium and relative decrease in chloride concentrations (or vice versa). Either of these coincidences is highly unlikely.

This is one of the advantages of using different electrodes simultaneously: widely unacceptable readings can be discarded or corrected for by cross checking between the different electrode outputs.

---

1 Some experiments were performed in saline containing different potassium concentrations, NaH_2PO_4 being replaced by KH_2PO_4, for example.

The changes in potassium concentration can be computed from the data using the Nernst equation:

$$E_K = E_K^{ref} + \lambda \log [K] \tag{4}$$

$$\therefore \Delta E_K = \lambda \log \frac{[K]'}{[K]} \tag{5}$$

where E_K is the reading of the potassium electrode; E_K^{ref} is a reference value which depends on the choice of reference electrode and internal filling solution; λ is the slope of the potassium electrode (59 mV per decade); [K] and [K]′ are the potassium activities in the extracellular solution at the beginning and at the end of an experiment, respectively. $\alpha = [K]'/[K]$ is obtained directly from the change in potential ΔE_K. At constant ionic strength, the ratio of the concentrations is equal to the ratio of the activities:

$$\frac{(K)'}{(K)} = \frac{[K]'}{[K]} = \alpha. \tag{6}$$

The potassium flux J_K can then be computed:

$$J_K \simeq V(K) \frac{\alpha - 1}{\Delta t} \tag{7}$$

where V is the extracellular volume, (K) is the initial potassium concentration and Δt the time elapsed during the experiment. Normalizing this flux to a constant number of red cells:

$$\bar{J}_K \simeq \frac{V}{V_{RBC}^{isotonic}} (K) \frac{\alpha - 1}{\Delta t}. \tag{8}$$

If the experiment is made under isotonic conditions,

$$\frac{V}{V_{RBC}^{isotonic}} = \frac{1 - H}{H} \tag{9}^{2}$$

where H is the haematocrit of the cell suspension. Therefore,

$$\bar{J}_K \simeq \frac{1 - H}{H} (K) \frac{\alpha - 1}{\Delta t}. \tag{10}$$

For n consecutive experiments:

$$\bar{J}_{K,n} \simeq \frac{1 - H_1}{H_1} (K)_1 \, \alpha_1 \, \alpha_2 \ldots \alpha_{n-1} \frac{\alpha_n - 1}{\Delta t_n}. \tag{11}$$

An error analysis shows that α should be kept sufficiently large—say $\alpha \geqq 1.08$ (i.e.: $\Delta E_K \geqq 2.0$ mV). A worst possible relative error of $\pm 5\%$ is then obtained for $\bar{J}_K$. For consecutive experiments it is important that α be not too large—say $\alpha \leqq 1.7$ (i.e.: $\Delta E_K <$ 13.5 mV) and that not too many experiments be performed consecutively—say $n \leqq 6$. A maximum error of $\pm 7\%$ is then found for $\bar{J}_{K,n}$.

2 This expression is only approximate since H should be corrected for trapped extracellular volume. The error involved will be eliminated by dividing the fluxes by a reference value $\bar{J}_{K,1}$.

Results

Typical Experiments – Effect of Ouabain

Fig. 3 shows the results of a typical experiment. The graph consists of three distinct parts:

(1) 0 to 20 min. The electrodes are first allowed to stabilize in 40 cc of saline solution; all the readings are seen to become constant.

(2) 20 to 50 min. When 30 cc of packed red blood cells are added to the system, the pH electrode reading jumps by about 24 mV (pH 7.4 to pH 7.0). This is due to the acidity of the cells that have been stored in ACD.

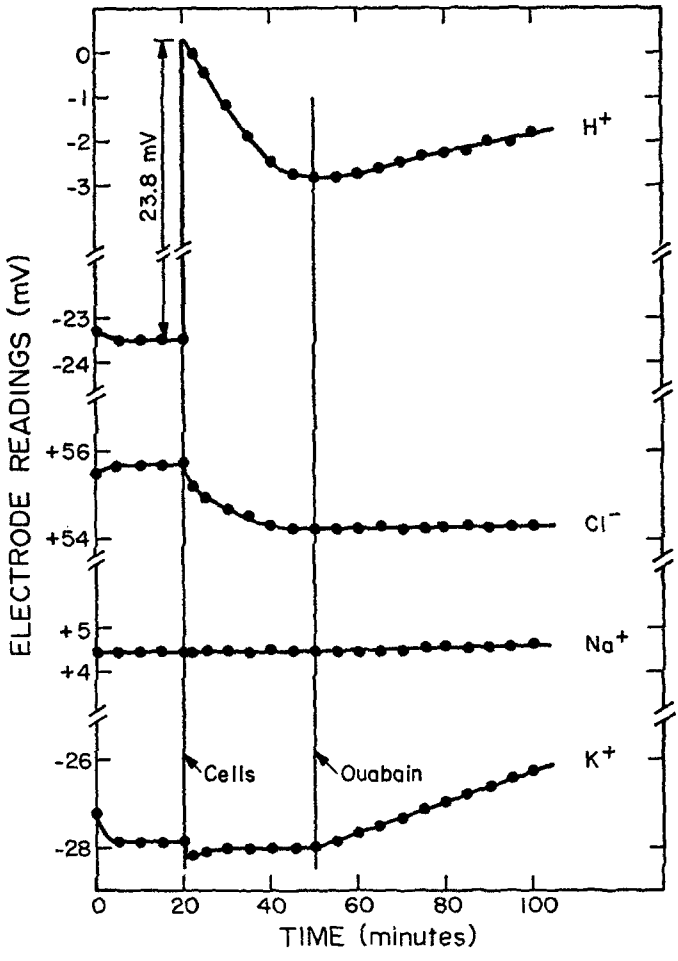

Fig. 3. Typical experiment. At time 20 min, 31.5 cc of packed red cells are added to 40 cc of isotonic buffered saline, yielding a suspension of 39% haematocrit. At time 50 min, 15 mg of ouabain are added to the system

The hydrogen ion concentration then decreases as the extracellular phosphate buffer exchanges with the intracellular chloride. This is seen by a decrease in the chloride electrode reading (corresponding to an increase in Cl^- activity). The following rise in the hydrogen ion concentration [3] is thought to be the result of metabolic activity of the red blood cell which produces lactic acid [25]. The maximum pH is always reached between 20 and 40 min after addition of the cells into the saline solution. Coincidentally, in this particular experiment, ouabain is added at about the time the pH reaches its maximum. The sodium and potassium electrodes give stable readings slightly different from those of the saline solution.

(3) 50 to 100 min. Addition of ouabain in the system has a unique effect of increasing the rate of change of the potassium readings. This is the expected result since the sodium-potassium pump is supposed to be poisoned. The amount of ouabain added is not critical as it is always in great excess of the minimum necessary to inactivate the active transport: about 10^{-4} M compared to 10^{-7} M reported in the literature [8].

Both sodium and chloride readings remain constant within the accuracy of the method. (The sodium reading increases by about 0.1 mV in 1 hr.)

Variations up to 100% in potassium fluxes were registered among different blood samples. Even on a unique sample, variations up to 15% were observed from one day to another. The difficulty in the reproducibility of the results was alleviated by varying the experimental conditions on the same blood, in the same day, in the course of a unique experiment.

The changes in ionic concentrations measured with the electrodes upon osmotic swelling or shrinking of the cells were found to be consistent with the well-known result that water crosses osmotically the membrane free of ions [21].

As the results obtained by Wilbrandt and Schatzmann [26] and then by Rothstein and co-workers [5, 11, 20] were thought to be rather unexpected, a series of experiments was performed to try to extend their results to the physiological range. Given amounts of isotonic sucrose solution being added to a suspension of ouabain-poisoned red blood cells, the outflux of potassium increases markedly. A plot of $\bar{J}_K/\bar{J}_K^{ref}$ *vs.* $(Cl)_i/(Cl)_o$, is given in Fig. 4. $\bar{J}_K^{ref}$ is the potassium outflux when the red cells are suspended in isotonic saline at pH = 7.0. The chloride ratio is then approximately 1 [6]. For the other cases $(Cl)_i/(Cl)_o$ is computed from the changes in intracellular and extracellular aqueous volumes: $(Cl)_i/(Cl)_o \simeq V_o/V_o^{ref} \times V_i^{ref}/V_i$. The curve

3 It should be noted that this variation in pH is only of the order of 0.05 units for the whole experiment.

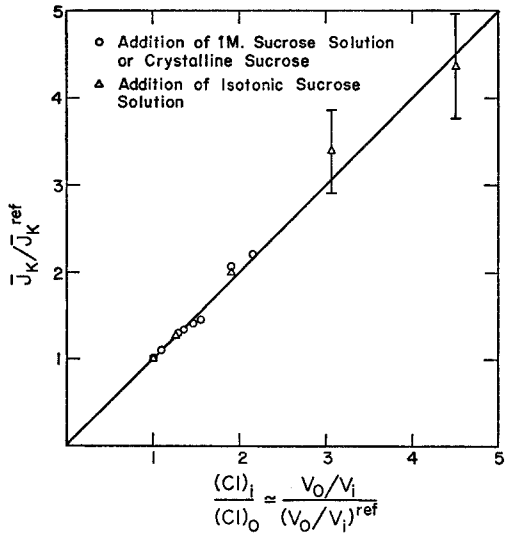

Fig. 4. Variations of the passive potassium efflux from red blood cells as a function of the chloride ratio. The osmolarity was increased up to 520 milliosmols in experiments where the chloride ratio was modified by addition of a hypertonic sucrose solution. The figure summarizes the results of several different experiments. To allow the comparison on a single figure, the fluxes have been normalized by division with the value obtained under isotonic conditions

$\bar{J}_K/\bar{J}_K^{ref}$ *vs.* $(Cl)_i/(Cl)_o$ is seen to follow the identity line. Therefore, the potassium flux $\bar{J}_K$ is proportional to the chloride ratio $(Cl)_i/(Cl)_o$.

To gain some insight into the preceding phenomenon, experiments were performed where concentrated sucrose solution or even crystalline sucrose was added directly to the cell suspension. The surprising result is that the increase in potassium outflux varies in the same way with the ratio $(Cl)_i/(Cl)_o$ as in the experiment where isotonic sucrose is added to the cell suspension. This is shown in Fig. 4 where the points obtained from both kinds of experiments are seen to follow the same identity line. Such a result is surprising since the addition of hypertonic sucrose solution shrinks the cells and thus increases the intracellular potassium concentration (up to 70% increase for experiments where the osmolarity was brought up over 500 milliosmols). There are two possible explanations for this apparent independence of $\bar{J}_K$ and $(K)_i$: either the increase in intracellular concentration has indeed no effect on the potassium outflux or its effect is balanced out by the changes in membrane permeability due to the increase in osmolarity.

To test the second explanation one can study the influence of changes in osmolarity at constant intracellular concentration. The addition of glucose allows such a study since it penetrates the red cells leaving the

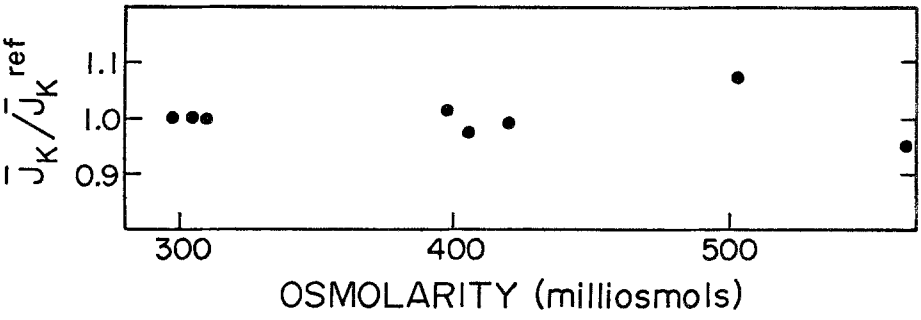

Fig. 5. Effect of osmolarity on the passive potassium efflux from red blood cells. The osmolarity is changed by addition of glucose

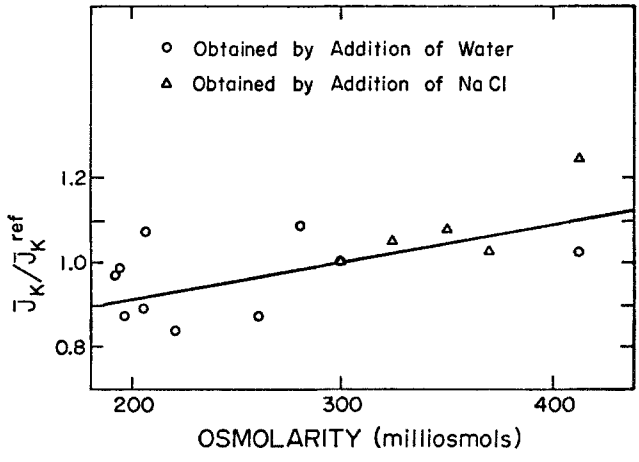

Fig. 6. Effect of changing the salt concentration on the passive potassium efflux from red blood cells

intracellar volume unchanged. After each addition of glucose a transient fall in extracellular concentrations is obtained due to the shrinkage of the cells. A corresponding transient increase in potassium outflux is also seen. As glucose equilibrates on both sides of the cell membrane the potassium outflux returns to its original value. Fig. 5 summarizes the results of such experiments: increasing the osmolarity by addition of glucose leaves the steady potassium outflux essentially unchanged.

From the results of the previous experiments, it appears that the potassium outflux in ouabain-poisoned red blood cells is independent of the intracellular potassium concentration and a unique function of the total potential difference across the cell membrane (i.e., the ratio $(Cl)_i/(Cl)_o$). An interesting test of this hypothesis would be obtained by varying the intra-

cellular concentrations while maintaining a constant ratio $(Cl)_i/(Cl)_o$. Such a condition can be approximated by the addition of water or sodium chloride in the suspending medium. Although these additions change the ionic strength, it is hoped that its influence is small enough not to mask the expected result; i.e., no change in the potassium outflux. The experiments summarized in Fig. 6 are not quite conclusive. Transient effects on the red cell membranes due to osmotic shock and experimental difficulties with the electrodes upon sudden changes in ionic strength probably concur in scattering the data. However, the results corroborate somewhat the hypothesis: varying the intracellular concentrations by $\pm 33\%$ does not change the potassium outflux by more than $\pm 15\%$.

Discussion

Direct use of electrodes in red cell suspensions is likely to create artifacts such as those discussed in Materials and Methods. It is somewhat of an art to obtain reliable data. From time to time the electrodes simply fail and a whole series of experiments has to be discarded. However, as the results of a typical experiment show, the method seems to work as well as can be expected: sodium, chloride and hydrogen ions can be controlled continuously and the small potassium outflux induced by addition of ouabain can be measured with reasonable accuracy.

The experiments where isotonic sucrose is added to the cell suspension confirm the results of Rothstein and co-workers [5, 11, 20]: the main parameter determining the rate of potassium efflux from red cells suspended in low ionic strength media is the potential difference across the membrane. However, Donlon and Rothstein [5] proposed that, close to the normal ionic strength, the potassium outflux depended linearly on the membrane potential. From our experiments, the flux seems to depend linearly on the chloride ratio and, thus, exponentially on the membrane potential. Fig. 7 shows a plot of the actual data obtained by Donlon [4]. It appears that the so-called linear dependence is more likely exponential. The line drawn on the graph corresponds to the equation that we obtained: $\bar{J}_K = \text{constant} \times (Cl)_i/(Cl)_o$ taking an arbitrary point of reference among the data. Fig. 7 also corroborates the results we obtained in experiments with hypertonic sucrose solutions: no matter whether the sucrose solution is isotonic or hypertonic, the potassium outflux is a unique function of the total membrane potential.

Using the data of Cotterrell and Whittam [2], one can study unidirectional potassium fluxes *vs.* the chloride ratio as shown in Fig. 8. The net

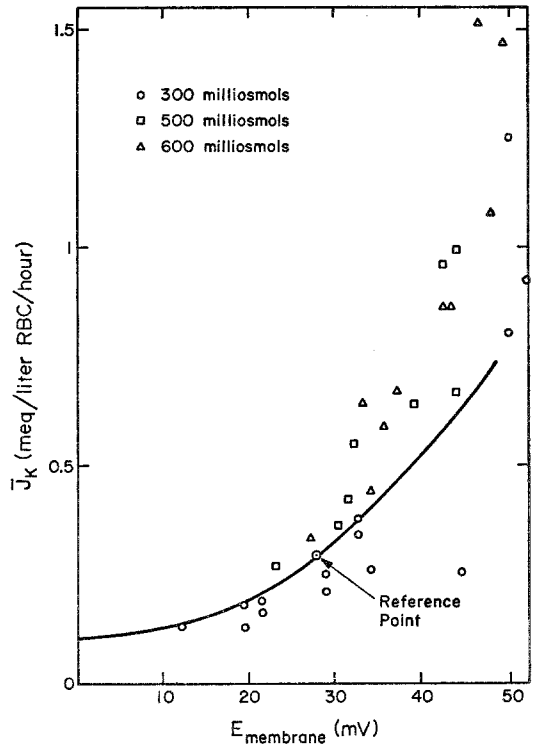

Fig. 7. The potassium efflux from red blood cells as a function of the membrane potential. (The membrane potential is modified by addition of a sucrose solution.) Data taken from Donlon [4]

flux is found to be proportional to the chloride ratio from $(Cl)_i/(Cl)_o = 0.75$ to $(Cl)_i/(Cl)_o = 1.5$. This is an especially interesting result as Cotterrell obtained the data by replacing the extracellular chloride with EDTA. The extracellular ionic strength was thus slightly increased, contrary to what happened in Donlon's [4] experiments as well as those reported here. The possibility that changes in extracellular ionic strength could have accounted for the increase in potassium efflux has then to be discarded.

The lack of influence of osmolarity, per se, on potassium outflux demonstrated by the glucose experiments is in agreement with the results obtained by Donlon [4] for the addition of glycerol which, like glucose, penetrates the red cell. Because of results with various sucrose solutions at ionic strength between 0.2 and 20 mM, Donlon [4] and Donlon and Rothstein [5] concluded that hypertonicity has a definite effect on membrane permeability to potassium. However, the addition of glycerol failed to demonstrate such an effect.

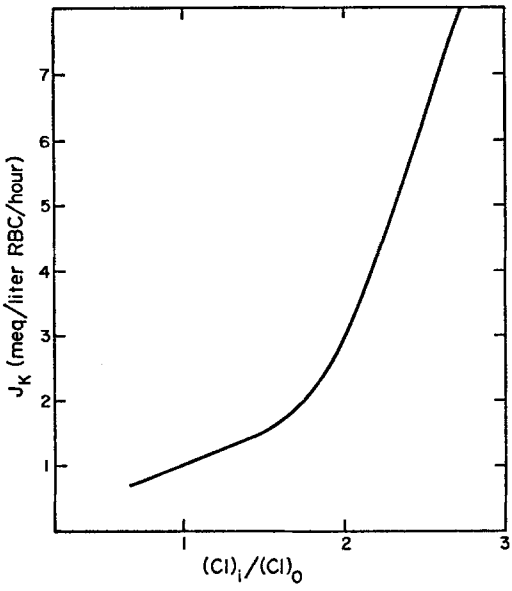

Fig. 8. The net potassium efflux from red blood cells as a function of the chloride ratio. (The chloride ratio is modified by replacing chloride with EDTA.) The curve is obtained from the data of Cotterrell and Whittam [2]

Although the experiments where sodium chloride or water are added to the cell suspension are not quite conclusive, the average result of the scattered data tends to show a slight increase in potassium outflux with increasing ionic strength. This would be in agreement with the results obtained by Passow [16] at very high ionic strength. Inasmuch as the ionic strength effect, per se, could explain the changes in potassium outflux, the data of Fig. 6 support the hypothesis that changes in intracellular potassium concentration do not affect the net flux of potassium.

By decreasing the extracellular pH, one can increase the chloride ratio across the red cell membrane (or vice versa) without changing any of the cationic concentrations. No experiment of this sort has been presented here because, in addition to modifying the chloride ratio, changing the pH is likely to affect any existing transport mechanism. The literature results on this subject are somewhat contradictory; the most accepted one is that changing the external pH from 6.0 to 8.0 has little effect on the potassium flux from red cells suspended in normal physiological saline [2, 16]. This could be due to a balance between a decrease in driving force (chloride ratio) and an increase in permeability (decrease in fixed cationic charge within the membrane, for example) as the pH increases.

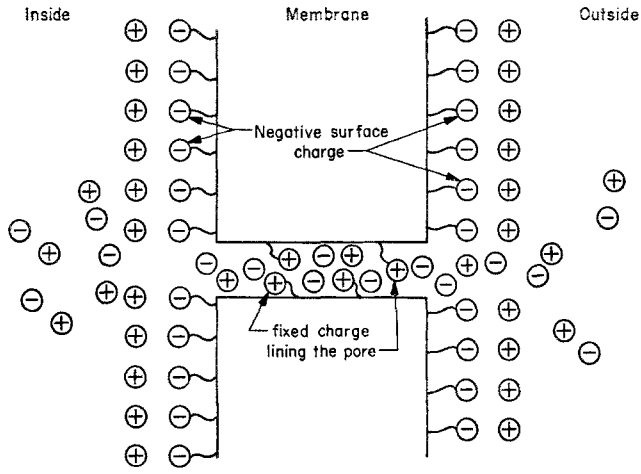

Fig. 9. Diagram of a hypothetical model for the red cell membrane that could make the potassium efflux independent of intracellular concentrations. This model is disproved in the Discussion

The principal results of this investigation can be summarized as follows: The main parameter determining the outflux of potassium induced by ouabain in red cells near physiological conditions is the total membrane potential. The potassium outflux appears to be independent of intracellular concentrations and proportional to the chloride ratio. Neither changes in osmolarity or in ionic strength greatly affect this potassium outflux.

These results are in sharp contrast to the usual predictions of the fixed-charge hypothesis. A saturation is predicted for the flux of counter ions when their bulk concentration is lower than the fixed-charge concentration in the membrane, but not for the co-ion [7]. One way to reconcile the fixed-charge hypothesis with the independence of the potassium flux and the intracellular potassium concentration is to consider as separate the rate-determining barrier for diffusion and the sites controlling the intramembrane concentration, much in the same way as Passow [15, 16] has suggested for anions. For example, Fig. 9 illustrates such a model. The negative charges on the surfaces of the membrane fix the total cation concentrations in the neighborhood of the membrane. The positive charges lining the pores control the diffusion of cations under fixed boundary conditions. It is not clear how such a model could account for the proportionality of the potassium flux and the chloride ratio: diluting the suspension with isotonic sucrose solution should have little effect on the boundary condition at the inner surface of the membrane. The potassium efflux should thus remain about

constant upon addition of isotonic sucrose as long as the extracellular potassium concentration is much lower than the intracellular one. This is in disagreement with the experimental results.

Other mechanisms such as the mediation of a carrier or negatively charged pores can be saturated and make the potassium outflux independent of the intracellular concentrations. From the observed differences between the characteristics of the anionic and cationic permeabilities, it is quite probable that they correspond to different transport routes; for example, specialized pores or carriers. Indeed from the results of their experiments with chemical modifiers of the membrane, Knauf and Rothstein [9] have hypothesized the existence of those two distinct permeability barriers for anions and cations.

For membrane potentials between 40 and 160 mV, Rothstein and co-workers [5, 11, 20] found that the observed effects of concentrations, pH and temperature on the cationic fluxes supported the proposition that cationic permeabilities of the red cell are controlled by fixed positive charges such as $R\text{-}NH_3^+$. One can then postulate that for membrane potentials between 40 and 160 mV the main part of the cationic fluxes uses the same pathways as those normally used by anions. This phenomenon would parallel the increased potassium efflux observed with the addition of DNFB [9, 17].

It then appears that two distinct mechanisms, say A and B, would be responsible for the passive cationic fluxes. A would be the main route for cationic fluxes near physiological conditions. It would be saturated and its characteristics would be such that the potassium flux would depend exponentially upon the membrane potential and be relatively independent of pH or concentrations. B would correspond to a cationic leakage through the route normally used by anions and be comparatively small under physiological conditions. The sum of the two cationic fluxes mediated by A and B would yield curves with sharp inflections such as those obtained by Rothstein and co-workers [5, 11, 20]. A would dominate for very low or very high potentials ($V_m < 40$ mV or $V_m > 160$ mV) while it would be the opposite in the middle range. The curves would be, for example, the sum of an exponential and a straight line. Although direct extrapolation of the curve of Fig. 7 to potential around 160 mV yields values about twice as large as those actually observed, this could be explained by the effect of decreasing the extracellular ionic strength to values like 10^{-4} M.

The interpretation of Cotterrell and Whittam's [2] experiments is not quite straightforward since it is not clear what the effect of large concentra-

tions of a strong complexing agent such as EDTA will be on the erythrocyte membrane. However, it can be simply postulated that, in addition to the membrane potential effect as it is observed in low ionic strength cell suspensions, EDTA will neutralize some of the positive charges controlling the anionic channels thus increasing cationic fluxes through pathway *B* much more readily than in our experiments. This would explain why the linear dependence of the potassium efflux upon the chloride ratio breaks down for a membrane potential around 11 mV $[(Cl)_i/(Cl)_o \simeq 1.5]$ in Fig. 8 while it seems to hold for potentials up to 30 mV in Fig. 7.

Conclusions

The experiments herein presented demonstrate the feasibility of using on specific electrodes to measure ionic fluxes in red cell suspensions. The main results can be summarized as follows: (1) The potassium efflux induced by ouabain in human erythrocytes near physiological conditions is ndependent of the intracellular concentrations of potassium (in the range 0.1 M to 0.3 M, approximately); (2) it is proportional to the chloride ratio for values from 1 to 5 (0 to 40 mV); (3) it is independent of osmolarity in the range 200 to 600 milliosmols; (4) it increases slightly with ionic strength.

These results are in fair agreement with those obtained by Rothstein and co-workers [5, 11, 20], using a conductivity method and with those obtained by Cotterrell and Whittam [2] using radioactive tracers.

The fixed-charge hypothesis, in its usual form, does not account for the above results. To explain all the experimental data available on the passive transport of potassium, two parallel routes for the efflux of potassium might be invoked: a special cationic pathway and a leakage through the channels normally used for anionic transport.

This work was supported in part by a grant from the U. S. Public Health Service, No. HE 08977.

References

1. Barr, L. 1965. Membrane potential profiles and the Goldman equation. *J. Theoret. Biol.* **9**:351.
2. Cotterrell, D., Whittam, R. 1971. The influence of the chloride gradient across red cell membranes on sodium and potassium movements. *J. Physiol.* **214**:509.
3. Deuticke, B. 1970. Anion permeability of the red blood cell. *Naturwissenschaften* **57**:172.

4. Donlon, J. A. 1968. Passive cation efflux from human erythrocytes suspended in low ionic strength hypertonic media. Ph. D. Thesis. University of Rochester, Rochester, New York.

5. Donlon, J. A., Rothstein, A. 1969. The cation permeability of erythrocytes in low ionic strength media of various tonicities. *J. Membrane Biol.* **1**:37.

6. Gary Bobo, C. M., Solomon, A. K. 1968. Properties of hemoglobin solutions in red cells. *J. Gen. Physiol.* **52**:825.

7. Helfferich, F. 1962. Ion Exchange. McGraw-Hill Book Co., Inc., New York.

8. Hoffman, J. F., Ingram, C. J. 1968. Cation transport and the binding of *T.* Ouabain to intact human red blood cells. *In:* Stoffwechsel und Membranpermeabilität von Erythrocyten und Thrombocyten. E. Deutsch, E. Gerlach and K. Moser, editors. p. 420. Georg Thieme Verlag, Stuttgart.

9. Knauf, P. A., Rothstein, A. 1971. Chemical modification of membranes I & II. *J. Gen. Physiol.* **58**:190.

10. La Celle, P. L., Passow, H. 1966. Effects of variations of pH and ionic strength on anion permeability of the erythrocyte membrane. *Pflüg. Arch. Ges. Physiol.* **291**:R 15.

11. La Celle, P. L., Rothstein, A. 1966. The passive permeability of the red blood cell to cations. *J. Gen. Physiol.* **50**:171.

12. Lepke, S., Passow, H. 1971. The permeability of the human red blood cell to sulfate ions. *J. Membrane Biol.* **6**:158.

13. Maizels, M., Patterson, J. H. 1940. Suvival of stored blood after transfusion. *Lancet* **239** II:417.

14. Morel, F. M. 1971. A study of passive potassium efflux from human red blood cells using ion specific electrodes. Ph. D. Thesis, Part I. California Institute of Technology, Pasadena, California.

15. Passow, H. 1965. Passive ion permeability and the concept of fixed charges. *Proc. XXIII Int. Physiol. Sci. Congr.*, Tokyo. *Int. Congr. Sci., Excerpta Med., Amst.* **87**:555.

16. Passow, H. 1969. Passive ion permeability of the red blood cell. An assessment of the scope and limitations of the fixed charge concept. *Prog. Biophys. Mol. Biol.* **19**: Pt. II, 423.

17. Poensgen, J., Passow, H. 1971. Action of 1-fluoro-2,4-dinitrobenzene on passive ion permeability of the human red blood cell. *J. Membrane Biol.* **6**:210.

18. Ponder, E. 1948. Haemolysis and Related Phenomena. Greene and Stratton, New York.

19. Post, R. L., Albright, C. D., Dayani, K. 1967. Resolution of pump and leak components of sodium and potassium ion transport in human erythrocyte. *J. Gen. Physiol.* **50**:1201.

20. Rothstein, A. 1968. Membrane permeability of erythrocytes. Stoffwechsel und Membranpermeabilität von Erythrocyten und Thrombocyten. E. Deutsch, E. Gerlach and K. Moser, editors. p. 407. Georg Thieme Verlag, Stuttgart.

21. Savitz, D., Sidel, V. W., Solomon, A. K. 1964. Osmotic properties of human red cells. *J. Gen. Physiol.* **48**:79.

22. Teorell, T. 1953. Transport processes and electrical phenomena in ionic membranes. *Prog. Biophys. Biophys. Chem.* **3**:305.

23. Tosteson, D. C. 1959. Halide transport in red blood cells. *Acta Physiol. Scand.* **46**:19.

24. Tosteson, D. C., Cook, P., Andreoli, T. E., Tieffenberg, M. 1967. The effect of valinomycin on potassium and sodium permeability of HK and LK sheep red cells. *J. Gen. Physiol.* **50**:2513.

25. Whittam, R. 1964. Transport and Diffusion in Red Blood Cells. Williams and Williams, Baltimore, Maryland.

26. Wilbrandt, W., Schatzmann, H. J. 1960. Changes in the passive permeability of erythrocytes in low electrolyte media. *In:* CIBA Foundation Study Group Symposium No. 5. Regulation of the Inorganic Ion Content of Cells. London, Churchill, p. 34.