QUANTITATIVE RELATIONSHIPS BETWEEN THE CONCENTRATION OF PROTEINS AND THE CONCENTRATION OF K^+ AND NA' IN RED CELL GHOSTS

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The concentration of K^+ accumulated in and Na^+ extruded from red blood cell ghosts are quantitatively correlated with the total protein contents of the ghosts with a linear correlation coefficient of +0.80. The straight line derived from the 43 sets of data on K^+ accumulated in the presence of varying ghost proteins, when extrapolated to the protein content of a normal red blood cell, yields a total K^+ content close to that actually found in normal red blood cells. This quantitative relationship agrees with the prediction of the AI hypothesis that there should be a stoichiometric relation between the K-adsorbing protein(s) and the concentration of K^+ accumulated. In addition the data suggests that in red blood cells the protein providing most of the K^+ adsorbing sites is hemoglobin. The apparent equilibrium distribution coefficient, or ρ-value, of Na^+ dissolved in the ghost water at different protein concentrations and in intact red blood cells follows a pattern similar to that seen in solutions of gelatin and of oxygen-containing linear polymers.

INTRODUCTION

According to the membrane-pump theory, the bulk of cell water and ions (primarily K^+) exist in a free state. Pumps located in the cell membrane maintain the asymmetrical distribution of K^+ and Na' across the cell surface. In the sixties and early seventies, one could often read in the literature, statements to the effect that red cell ghosts are able actively to pump both Na' and K' ions against concentration gradients. These statements, when seen in the light of the not infrequently expressed opinion that red cell ghosts are pure cell membranes (see Ling, 1984, p. 129) have led many to believe that the membrane-pump theory of the living cell had received strong experimental support from the studies of red cell ghosts.

Concluding a careful literature search, Freedman wrote in 1973, "At present a widespread misconception prevails that net movements of sodium and potassium against an electro-chemical gradient have been well characterized with RBC ghosts. Since no unequivocal evidence exists in the literature, net pumping of sodium and potassium by red cell ghosts cannot be used to support the membrane theory.. ."(Freedman, 1973a, p. 613). Yet Freedman was soon to announce his success in demonstrating what he believed that others before him had failed to demonstrate (see, however, Hashimoto and Yoshikawa, 1963; Ling, 1984). He showed that red cell ghosts in the presence of ATP could indeed recover part of the K' lost during hemolysis and extrude Na^+, each against a concentration gradient (Freedman, 1973b, 1976).

A key statement in Freedman's paper was, "Since the water content was constant at 95%, the conclusions do not depend on whether the ion contents are expressed on a wet-weight or water basis." (Freedman, 1976, p. 991). The implication of this statement was that these ghosts that had been shown ap-

parently to be actively transporting K' vs. Na^+ against concentration gradients contained little more solids than the cell membranes. However, subsequent electron microscopic studies (Ling and Balter, 1975; Hazlewood, Singer and Beall, 1979; Ling and Tucker, 1983) revealed that the ghosts prepared by the method of Freedman and other similar methods were solid rather than hollow. Ling and Tucker (1983) then went on to show that while solid ghosts prepared by the method of Freedman do indeed transport K' and Na' against concentration gradients, hollow (white) ghosts, prepared by the method of Marchesi and Palade (1967) (and of Dodge et al., 1963) do not, even though they have intact membranes and membrane K', Na^+ activated ATPase.

Mature human red blood cells contain no DNA and RNA; lipid and phospholipid contents of each red blood cell remain essentially unchanged even after the much more extensive removal of the intracellular proteins by the method of Dodge et a1 (1963). Therefore, the electron-dense materials revealed in the EM plates of red cell ghosts prepared by the much gentler Freedman procedure must be primarily proteins. Ling and Tucker's work described above had in fact demonstrated the ***requirement of intracellular proteins for the selective uptake of K^+ and the extrusion of Na^+ in the resealed red cell ghosts.***

Our next question is, "How and why are these intracellular proteins essential for the accumulation of K' and extrusion of Na'?" The dependency of ion transports on intracellular proteins is not predicted by the membrane-pump theory, according to which the levels of K^+ and Na' are determined only by the cell membrane and pumps within the cell membrane. In contrast, this dependency agrees with the association-induction (AI) hypothesis, according to which selective accumulation as well as Na' extrusion are due to proteins within the cell (Ling, 1984). Thus K' accumulates in the cells because it is preferentially adsorbed on the p- and γ-carboxyl groups belonging respectively to aspartic and glutamic acid residues of certain intracellular proteins. The exclusion of Na' also depends on certain intracellular proteins, called "matrix proteins": Matrix proteins exist, under suitable conditions, in the extended form in which their NHCO groups are directly exposed to and polarize the bulk of cell water. Water in the state of polarized multilayers exhibits decreasing solubility for molecules and hydrated ions of increasing size and complexity (the size rule). Large hydrated ions like Na' (and K') are therefore found in lower concentration in the cell water than in the external medium. Thus the low equilibrium distribution coefficient, or q-value, of a solute like Na' in the cell water depends on proteins with extended chains and exposed NHCO groups. The low q-value for Na' in turn accounts for the low concentration of this ion found in most living cells.

What intracellular proteins provide the β- and γ-carboxyl groups for selective K' adsorption and what proteins serve the role of matrix proteins may vary with cell types. It is also uncertain whether the K^+ adsorbing and water polarizing functions are served by different or the same protein(s). However, in the AI hypothesis, K' accumulation and Na' extrusion are due to two different mechanisms, and the theory does not predict a simple one-to-one relation between K^+ reaccumulated in and Na' extruded from red cell ghosts.

In this communication, we report further experiments designed to understand more about the relation between the intracellular proteins and the levels of K' and of Na' in human erythrocyte ghosts.

MATERIALS AND METHODS

All blood used was freshly drawn from adult donors, both male and female. The

basic methods of ghost preparation, resealing, separation into Type II and Type III on a sucrose cushion, and incubation were those described by Freedman (1973b, 1976). However, attention to certain details is essential in generating reproducible results, so we will describe the procedures in some detail.

1. ***Washing:*** After removal of the plasma and buffy coat by centrifugation, the red cells were washed at room temperature ***twice*** in an equal volume of RBC washing solution (NaCl, 140 mM; KCl, 10 mM; $MgCl_2$, 2 mM; Tris HCl, 10 mM; Na_2EDTA, 0.1 mM; pH, 7.7) and spun down at 4000 g. at room temperature. The twice-washed cells were then taken up in a syringe attached to a 16 gauge needle or catheter.

2. ***Lysing:*** The lysing solution contained 5 mM Na_2ATP; 1 mM L-cysteine, 7 mM $MgCl_2$, 10 mM Tris HCl, and 0.1 mM Na_2EDTA. The ATP was added to the lysing solution immediately before lysing and the pH of the ATP containing lysing solution adjusted to 4.6. 100 ml of lysing solution was chilled to from 0° to 1°C in a 250 ml fleaker (cylindrical beaker with constricted neck) in an ice bath containing mixture of water and finely crushed ice. While the lysing solution was stirred with a magnetic stirring bar (25 X 8 mm.), spinning at the rate of 360 revolutions per minute, 10 ml of the washed cells was rapidly injected into the stirred lysing solution with the tip of the syringe needle immersed just under the surface of the lysing solution. A pH electrode in the lysing solution monitored the pH. Adjustment of the pH to 6.0 was made with 2 N NaOH and 0.1 N HCI. Lysing continued at pH 6.0 for 10 minutes at 0° to 1°C.

3. ***Resealing:*** With a similar syringe and 16 gauge needle we introduced 10 ml (i.e., the same volume as that of the washed RBC lysed) of resealing solution (NaCl, 0.5 M; KCl 15 mM; sucrose, 2.0 M; pH, 8.4), adjusted to a pH of 7.1 with 2 N NaOH and continued stirring at 0° to 1°C for 1 minute. The fleaker was then covered with parafilm and moved to a 37°C water bath and incubated for 50 minutes with gentle shaking (60 excursions per minute, each excursion 1 inch).

4. ***Washing:*** Ghosts were spun down and then washed 2 or 3 times for 15 min., each time at 27,000 g (15000 rpm in a Sorval PR-2 with an SS34 rotor) in ghost wash solution (NaCl, 5.0 mM; KCl, 10 mM; MgCl, 2 mM; Tris HCl, 10 mM; Na_2EDTA, 0.1 mM; sucrose, 180 mM, pH, 8.3), until the supernatent was clear.

The following step may be either one of two alternatives: In the standard Freedman procedure one proceeds to ghost separation (Step 4A). In the "simplified procedure" one proceeds directly to incubation (Step 5). In either procedure, a set of initial samples of the spun down ghosts and of the supernatent of the last wash solution are taken for K^+ and Na^+ analysis.

4.A. ***Ghost separation:*** Washed ghosts were resuspended in ghost wash solution to make a 60% ghost separation (v/v). With a pipette 5 ml of this suspension was carefully layered on a 25 ml sucrose-buffer cushion in a 50 ml centrifuge tube. The sucrose buffer contained sucrose (43%, w/v), Tris HCl (10 mM), and NaCl (25 mM) at pH of 7.0. The suspension was spun at 12000 rpm (17000 g) for 1 hour. The red "disc" floating on top of the sucrose cushion is called Type II ghosts and the pellicle at the bottom of the sucrose cushion is called Type III ghosts (Bodeman and Passow, 1972). Type II ghosts were carefully pipetted off the top, the sucrose buffer poured off, and Type III ghosts collected. Type II and Type III ghosts were resuspended in ghost wash solution, and spun down at 15000 rpm (15 min.). A set of initial samples, were spun down and some supernatent saved. Ghosts were then treated as described under Step 5.

5. ***Incubation:*** *A* known volume of the washed, unseparated ghosts directly from

Step 4 or Type II and Type III ghosts from Step 4A were taken up in a graduated pipette with calibration to the tip and mixed with 4 volumes of incubation solution (NaCl, 50 mM; KCl, 10 mM; $MgCL_2$, 2 mM; Tris HCl, 10 mM; Na_2EDTA, 0.1 mM; inosine, 10 mM; adenosine, 10 mM; and sucrose, 160 mM) to make a 20% (v/v) suspension. The suspension was incubated in a water bath at 37°C with gentle shaking (30 excursions per min.; 1 inch displacements) and samples taken out after 18 hours of incubation.

6. ***Sample analysis:*** The number of aliquots taken for each sample are duplicates for dry weight determination and triplicates for ion analysis. With a Pasteur pipette 0.4 ml Eppendorf microcentrifuge tubes were filled with the ghost suspension and covered with the attached caps. For separating the ghosts from the supernatent, we used in our earlier experiments microcentrifuge tubes held in adapters in a Sorvall SS-34 rotor and spun at 4°C for 15 min. at 15000 rpm (roughly 19000 g). In later work an Eppendorf 5412 microcentrifuge was used to spin down the ghosts at 15000 rpm (15600 g.) for 3 min. at room temperature. No difference could be detected between these two procedures. The Eppendorf microcentrifuge requires less time and is therefore preferred.

With a Pasteur pipette the supernatent solution was carefully removed from each microcentrifuge tube (without disturbing the ghost pellet) and the samples of supernatent solution from each set of duplicate or triplicate samples were combined to yield a total of 0.5 to 1 ml needed for ion assays. With a new and clean single-edge razor blade, the centrifuge tubes were cut once just below the top surface of the ghost pellet and a second time just above the bottom of the ghost pellet. Using a rubber tubing that fits snugly onto the bottom of the cut microcentrifuge tube, one gently blows out the ghosts into an appropriate vessel described next.

Dry weight: Ghost samples were expelled into preweighed dried aluminum foil dishes (for milk analysis, A. H. Thomas, Philadelphia), and the loaded dishes weighed before and after drying in two steps: overnight drying in a warm room (40°C) and another 16 to 24 hours in an 100°C oven.

Ion analysis: Ghosts were expelled into preweighed Nalgene centrifuge tubes (12 ml capacity) and weighed again to obtain fresh

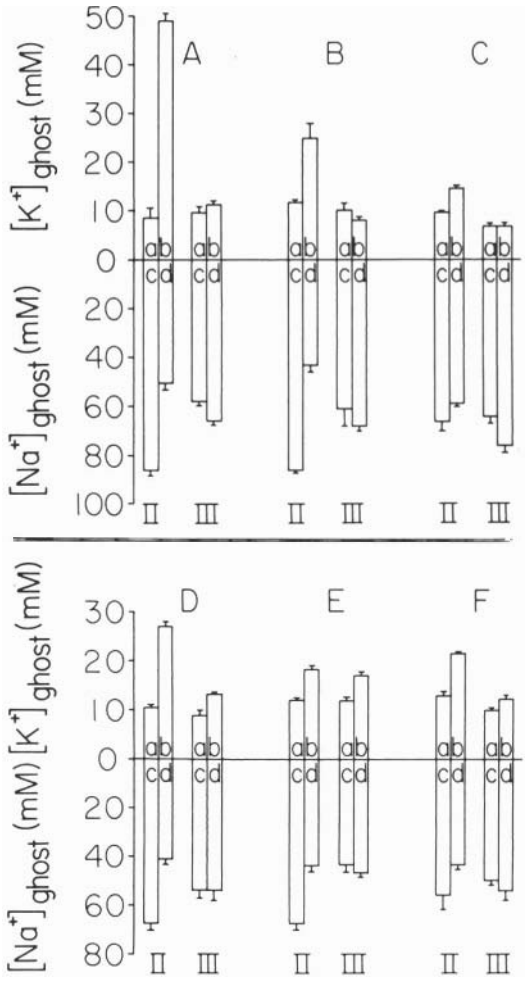

FIGURE 1. **K^+ and Na^+ contents in Type II and Type III red blood cell ghosts. A - F refer to six different blood donors. a, b refer to initial and final K^+ concentrations (18 hours) in the ghosts in' units of mM or mmoles/liter of ghost water. c, d refer to initial and final Na^+ concentrations (18 hours) in the ghosts in the same units.**

ghost weight. 3.0 ml of 0.3 M TCA was added and the tubes covered with parafilm and vortexed. They were then placed in a cold room without further disturbance for at least 24 hours to allow all proteins precipitated to settle down at the bottom of the tube. To make a 40 times dilution, a 0.20 ml aliquot of the clear extract was added to 7.8 ml of a 1.026 X UEB (universal extractive and radiation buffer) prepared by diluting a stock 10 X UEB which contains 970 mM LiCl, and 30 mM $NH_4H_2PO_4$ with ion-free distilled water. The K' and Na' contents of the samples were read against K' and Na' standards, containing 97 mM LiCl and 3 mM $NH_4H_2PO_4$ but no TCA (see below).

Supernatents: For Na' analysis, the supernatent was diluted 1001 times by adding 0.01 ml of the supernatent solution to 10 ml of 1.001 X UEB, which in addition to LiCl (97 mM) and $NH_4H_2PO_4$ (3 mM) also contained TCA (7.5 mM). The sample was read against Na' standards containing LiCl (97 mM), $NH_4H_2PO_4$ (3 mM), and 7.5 mM TCA. For K' analysis, the supernatent samples were diluted 201 times by mixing 0.05 ml of the superriatent to 10 ml of 1.005 X UEB containing 7.5 mM TCA, which is also present in all the K' standards at the same concentration.

K' and Na' in diluted samples were read on a Perkin Elmer Atomic Absorption Spectrometer Model 103.

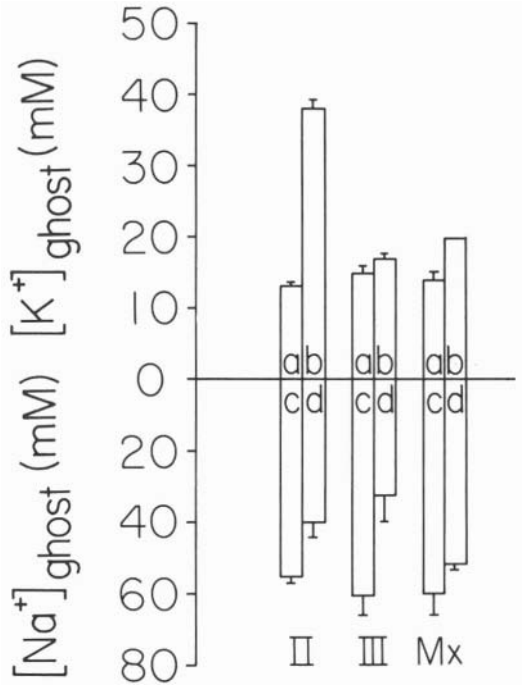

FIGURE 2. K^+ in and Na^+ contents in ghosts from a single donor. II and III refer to Type II and Type III ghosts as in Figure 1. Mx refers to resealed ghosts mixed with the sucrose cushion before spinning; all Mx ghosts sank to the bottom of the cushion. a, b, c, d have the same meaning as in Figure 1.

RESULTS

The underlying causes of the different behaviors of Type II and Type III ghosts. In a preceding paper of this series, Ling and Tucker (1983) confirmed the reports of Freedman that Type II red cell ghosts prepared by Freedman's modified Bodeman-Passow procedure could transport K' and Na^+ against concentration gradients. However, the extent of the K' accumulation and Na^+ exclusion varied considerably when blood from different donors was used. Type II ghosts (i.e., ghosts that were harvested on top of the sucrose cushion) were exclusively used in both Freedman's studies (1973a) and in the wcrk of Ling and Tucker (1983) referred to above. Figure 1 shows data from both Type II and Type III ghosts (collected from the bottom of the sucrose cushion) from seven different donors and treated in an identical manner. As a rule, the Type III ghosts exhibited no or little K' accumulation and no or little Na' extrusion and thus differed markedly from behavior of Type II ghosts of blood from the same donor.

One recalls that Bodeman and Passow (1972) originally introduced the sucrose gradient technique in order to sort out nonleaky (Type II) ghosts which do not admit sucrose and stay on top of the sucrose cushion, from

leaky (Type III) ones that do admit sucrose and fall to the bottom of the sucrose cushion. On the basis of his reasoning, one would expect that since a substantial part of the ghosts regularly comes through the sucrose cushion as Type III, the performance of the selected Type II ghosts (in regard to K^+ accumulation and Na^+ extrusion) should be considerably better than ghosts harvested directly after resealing without undergoing the sucrose cushion separation. We were puzzled by the fact that unseparated ghosts and Type II ghosts in fact performed equally well (see below). We then asked the question, "Could the different behaviors of Type II ghosts originate as a result of inadvertent exposure to the intensely hypertonic 43% sucrose cushion?" To test this idea, we divided the resealed ghost suspension from donor G into two parts. One part went through the usual layering procedure. The other part was not layered over the top of the sucrose cushion but was *mixed* with the 43% sucrose solution before centrifugation. While the ghosts layered over the sucrose cushion yielded the typical Type II ghosts on the top and Type III at the bottom, all the ghosts that were mixed with the sucrose solution sank to the bottom. Here samples of ghosts collected from the bottom of the cushion showed no or only weak K' accumulation and Na' exclusion much as the Type III ghosts do (Figure 2). The conclusion from this set of experiments is that the poor performance of the Type III ghosts is due, at least in part, to their being inadvertently mixed with the concentrated sucrose solution when the ghost suspension is layered over the top of the cushion. For this and other reasons to be given below, we decided that it would be better in most cases to eliminate the time-consuming sucrose cushion separation step.

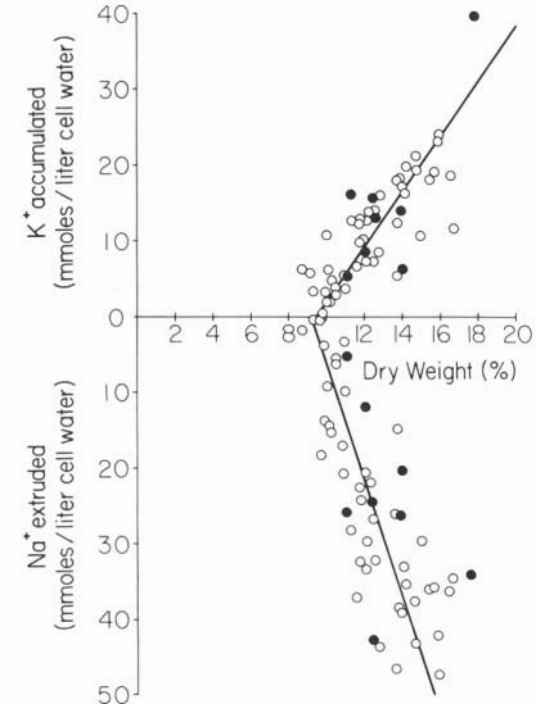

FIGURE 3. **K^+ accumulation in and Na^+ extrusion from unseparated ghosts prepared from blood of different donors and of the same donors (at times at least 6 weeks apart) are shown by empty circles and plotted against the total dry weights of the ghosts. Ordinate in mmoles/kilogram of ghost water (mM). Each data point represents the difference in K^+ or Na^+ concentration at the beginning of incubation and after 18 hours of incubation at 37°C. Straight lines were obtained by the method of least squares (see text for details).**

Relation between the total dry weight of the ghosts and the extent of K^+ reaccumulation and Na^+ extrusion. Using the simplified procedures without the step separating Type II and Type III ghosts, we studied the K^+ accumulation and Na' extrusion in 43 individual experiments. The procedures and solutions used were identical; the only variables were the genetic and physiological differences of ghosts prepared from blood of different donors. (Some donors contributed blood more than once; others only once.) The data shown in Figure 3 as empty circles were expressed as net accumulation (K') and net extrusion (Na') per liter of ghost water after 18 hours of incubation at 37°C following resealing and washing of the ghosts. Like the data shown in Figure 1, there is a wide varia-

tion among the 43 sets of data, ranging from very little accumulation and extrusion to pronounced accumulation and extrusion. As a rule, ghosts that accumulate more K, extrude more Na^+; ghosts that accumulate less K^+, extrude less Na'.

The abscissa of Figure 3 represents the total dry weight of the ghosts. The straight lines going through the data points were obtained by the method of least squares. For K, the intercept is 9.19% and the slope is 7.69. The correlation coefficient (r) is +0.81. The data show that neither K^+ accumulation nor Na^+ extrusion occurred at total dry weight below 8%. Put differently, the data show that no K' accumulation nor Na' exclusion against concentration gradients occurred in ghosts with a total water content higher than 92%. Indeed under no condition have we even observed ghosts to have a water content equal to or exceeding 92%. The reason for the existence of a limiting water content will be made clear in a following section.

The data derived from "unseparated" ghosts are shown in Figure 3 as empty circles. The seven sets of data points of K' accumulation and Na' extrusion from Type II ghosts shown in Figure 1 are also represented in Figure 3 but as solid circles. There is no major difference between the K' accumulation and Na' extrusion in the Type II ghosts and the unseparated ghosts. Like the data from the unseparated ghosts, the Type II ghosts also show widely different dry weights consistently above 8% and widely different water contents, all consistently below 92%. From these data, we conclude that the statement of Freedman mentioned in the Introduction to the effect that all (Type II) ghosts had a uniform water content of 95% is mistaken. Next we shall examine what the dry weight represents and how much of the dry weight is due to proteins.

The protein content of the ghosts. It is widely believed that most, if not all the lipids and phospholipids of mature human erythrocytes reside in the red cell membrane (Gorter and Grendel, 1925). This belief is supported by the similar content of lipids and phospholipids in intact washed erythrocytes (5.75 X

	Water Content	Sucrose Content	
Donor	%	mmoles per liter of cell water	% (grams per 100 ml ghost water)
S.A.	88.4±0.1	182.0±1.9	6.22
M.S.	89.3±0.2	174.4±1.7	5.96
L.F.	88.6±0.1	176.2±2.0	6.02
L.F.	86.7±1.0	175.5±5.8	6.00
mean ± S.E.			6.05±0.06

TABLE I. Sucrose concentration in unseparated red cell ghosts. Sucrose concentrations were analyzed chemically in TCA extracts of red cell ghosts from four donors and expressed in units of mmoles per liter of ghost water.

10^{-13} g/per intact erythrocytes); and in white ghosts prepared by the method of Dodge et al (1963) (5.89 to 6.00 X 10^{-13} g. per white ghost). Total lipid and phospholipid given by Ponder (1948, p. 120) in grams per 100 ml of intact red blood cells is 0.7 to 0.73%, averaging 0.72%; their salt ion content is 0.98% (K, 0.4%; Na, 0.05%; Cl, 0.18%; HCO_3, 0.1%; total PO_4, 0.25%) (Ponder, ibid). The salt ion content of the incubation solution is 1.11% (see Methods). From our own data, the average total weight of K, Na^+, and Cl^- in the ghosts is estimated at 6.29 ± 0.20 gm/liter of ghost water. Sucrose comprises 6.2% (w/v) of the "resealed lysing solution", 6.2% in the ghost wash, and 5.5% of the incubation solution and is by far the most prominent component of the solid matters in these solutions.

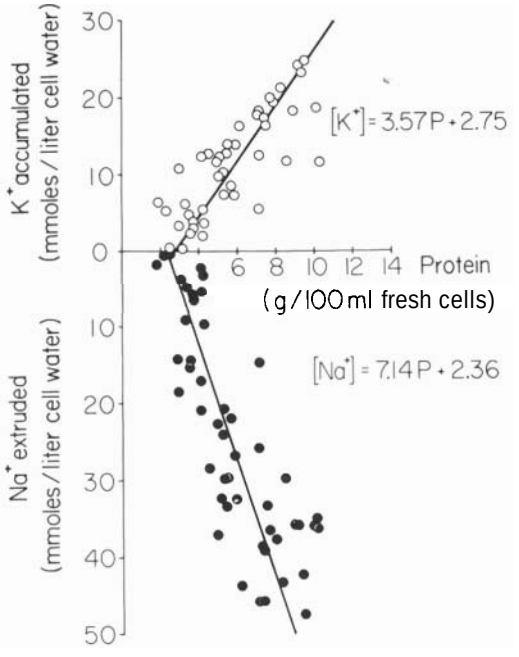

FIGURE 4. K^+ accumulation in and Na^+ extrusion from unseparated red cell ghosts against the total protein contents of the ghosts. Data points derived from the same experiments described in Figure 3. Straight lines were obtained by the method of least squares: $[K^+]_{accumulated} = 3.57$ [proteins] + 2.75. $[Na^+]_{extruded} = 7.14$ [proteins] + 2.36. Total protein contents were obtained by subtracting weights of lipids, phospholipids, salt ions, and sucrose from dry weights.

In view of the low permeability of the ghost membrane to sucrose (Freedman, 1973b), one may expect the sucrose content of the resealed ghosts after 18 hours of incubation to be close to 6%. An actual analysis of the sucrose contents of these ghosts, given in Table I, confirms this expectation. The average sucrose content of ghosts is 6.05% ± 0.06% in grams per 100 ml of ghost water.

From the above consideration, one concludes that after 18 hours of incubation the resealed ghosts contain, besides proteins, 0.72% lipids and phospholipids, 0.63% salt ions, and a varying amount of sucrose depending on the water content of the ghosts. Thus the total protein content (in %, w/v) can be obtained from the total dry weight (%, w/v) and water content (%, w/v) by the formula:

$$\text{protein content} = \text{dry wt.} - 1.35$$

$$(\text{water content} \times 0.0605).$$

With the aid of this formula, the data given in Figure 3 were recalculated and plotted in Figure 4 to represent net K' accumulation and net Na' extrusion both in units of mmoles per liter of ghost water, against the protein contents of the ghosts. Before proceeding to an analysis of the newly plotted data in Figure 4, let us return to the question of the observed 8% limiting dry weight for K' accumulation and Na' extrusion.

The white ghosts prepared by the method of Dodge et al represent ghosts with very little intracellular proteins. Six sets of dry weight values of these ghosts averaged (15.0 ± 1.6) × 10^{-13} gm. per ghost. Judging from the photographs provided by Dodge et al, these ghosts had retained more or less the size of the normal intact erythrocytes. The three sets of values for the mean corpuscular volume of these ghosts given by Dodge average 103 μ^3. The value of corpuscular volume collected by Ponder (1948, p. 79) from var-

ious sources is 87.5 μ^3. Thus the percentage dry weight of the white ghosts prepared by the method of Dodge et al should be $(15 \times 10^{-13})/(103 \times 10^{-12})$ or 1.46% according to the corpuscular value given by Dodge et al, or $15 \times 10^{-13}/87.5 \times 10^{-12} = 1.71\%$, according to the figures given by Ponder. Using an average 1.6%, one calculates a total water content in the white ghosts equal to $1 - .016 = 0.984$ or 98.4% water. Multiplying this figure with the average sucrose content in ghost water (6.05%), one estimates the sucrose content in white ghosts to be $6.05 \times .984 = 5.95\%$. The total dry weight of these white ghosts is thus $5.95 + 1.6\% = 7.55\%$. Since the white ghosts contain very little intracellular proteins, this figure sets the lower limit of the dry weight of ghosts that have been resealed, washed, and incubated for 18 hours in modified Ringer solution originally described by Freedman (1976). It is thus not surprising that the dry weights of the 50 sets of ghosts prepared by the Freedman procedure and shown in Figure 4 never fell below 8%. With the question of the 8% limiting dry weight clarified, let us return to explore further the significance of the data given in Figure 4.

The straight lines that go through the two sets of data in Figure 4 were also obtained by the method of least squares. The intercept of the K' data is now 2.75% ($r = +0.78$) and that of the Na' data, 2.36% ($r = +0.81$). The slope of the K' curve is 3.57 and that of the Na' curve is 7.14. Thus above a minimal limiting figure of 2% proteins, both K' accumulation and Na' exclusion are significantly correlated with the protein contents of the ghosts. For each 10 grams of additional intracellular proteins (per liter of fresh ghosts), there is an additional 3.57 mmoles K' accumulated in the ghosts and an additional 7.14 mmoles of Na^+ extruded from a liter of cell water. The relation between K' gained and Na' extruded is 1 to 2. However, for reasons to be given under Discussion too much emphasis should not be placed on the apparent stoichiometric relationship. The most significant point made here is that the movements of K^+ and Na' are not a simple one-for-one ion exchange phenomenon.

DISCUSSION

According to the AI hypothesis, each K' ion accumulated in the red cell entails one β- or y-carboxyl group of some cellular protein. There is, therefore, an anticipated stoichiometric relation between the molarity of K^+ accumulated and the concentration of the specific cellular proteins involved. However, the AI hypothesis by itself does not tell explicitly what intracellular protein(s) provides the β- and γ-carboxyl groups, and therefore what percentage of the total proteins comprises this specific K'-adsorbing protein(s). The extent of the predicted quantitative relation between intracellular protein contents and K' accumulation in a cell would vary, depending on the percentage composition of the K'-adsorbing protein in the total protein contents and on how this percentage composition changes with changes in the total protein contents of the cells. Thus for an average cell, the task of sorting out which protein binds K^+ may be very difficult. Fortunately, red blood cells offer a special advantage for this task because an overwhelmingly large proportion of the intracellular proteins is hemoglobin: A single red blood cell contains 29×10^{-12} gm. of hemoglobin per cell, while the non-hemoglobin content is only 0.78×10^{-12} gm./cell (Ponder, 1948, p. 121). Thus hemoglobin makes up $29/(29 + 0.78) = 97.3\%$ of the total intracellular proteins of the mature red blood cell while all the non-hemoglobin proteins put together make up less than 3%.

If all the amino acid residues in all the non-hemoglobin proteins of the red blood cells are exclusively aspartic acid residues, they will provide a maximum of $27/115 = 235$ mmoles of β-carboxyl groups per liter of

cells. Since the total protein content of red blood cell is about 35% (Ponder, 1948) the β-carboxyl groups provided by these hypothetical non-hemoglobin proteins are 235 X 0.35 = 82.3 mmoles/liter of fresh red blood cells in comparison with the actual total K content of the human red blood cells of 96 to 100 mmoles per liter of fresh cells (see below). Thus theoretically, these non-hemoglobin proteins may provide most of the K'-adsorbing anionic sites needed. In fact, it is highly unlikely that all these non-hemoglobin proteins are poly-aspartic acids. It is more likely however, that non-hemoglobin protein may contain, say, 35% of its amino acid residues in the form of aspartic and glutamic acid residue, as it is in, for example, tropomyosin which is known to be rich in these amino acids in its composition (Ling, 1962, p. xxviii). In that case, the non-hemoglobin protein can only offer a total of 82.3 X 0.35 = 28.8 mmoles of K adsorbing sites per liter of fresh cells. However, even this calculation is based on the unlikely assumption that all the fixed carboxyl groups are free to adsorb K. More likely, a substantial portion of the fixed carboxyl groups exist as partners in "salt linkages" (Perutz, 1978; Ling, 1984; Ling and Zhang, 1984). In that case, the maximum number of anionic adsorption sites provided by the non-hemoglobin proteins would be too few to account for the known level of K found in red blood cells. This deduction does not rule out the possibility that non-hemoglobin proteins may provide part of the K'-adsorbing sites. The deduction does suggest that hemoglobin, by far the most abundant protein in red blood cells, is likely to provide most of the K adsorbing sites.

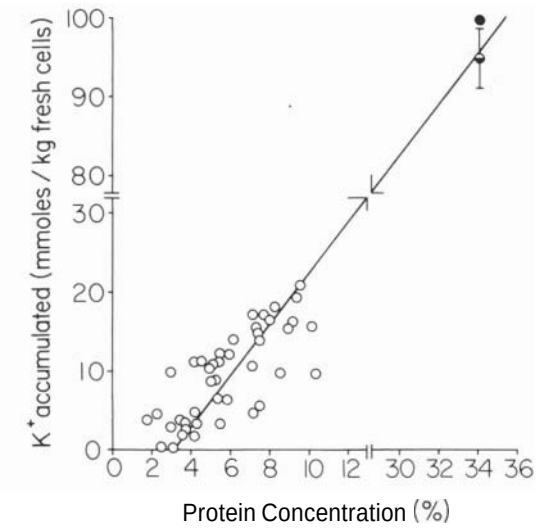

FIGURE 5. K⁺ accumulation in red blood cell ghosts and in intact red blood cells, in units of mmoles per kilogram of fresh ghosts or cells at different ghost or red cell protein concentrations in percentages (w/w). Data points of K⁺ accumulation in red cell ghosts are recalculated from those given in Figures 3 and 4. K⁺ contents of normal human red cells were taken from Freedman (1973b) (half-filled circle) and Ponder (1948) (solid circle). Protein contents of normal human red blood cells were taken from Ponder (1948, p. 120).

A chance for a test of the idea that hemoglobin offers most of the K⁺-adsorbing sites is provided by yet another plot of the data first given in Figure 2. Thus in Figure 5, the K accumulation data are recalculated once more and expressed in units of mmoles of K per gram of fresh cells. Again the method of least squares yields a relation

$$[K^+]_{accumulated} = 3.45\,[protein] + 3.12. \qquad (1)$$

Now if hemoglobin provides all or nearly all the K'-adsorbing sites and all hemoglobin in the red cell exists in the same conformation and within a similar microenvironment, one would anticipate that extrapolation of the straight line described by Equation 1 should "meet" with the K content of normal erythrocytes at their normal protein content. Figure 5 shows that this expectation was not too far from the mark. The half shaded circle represents average data given by Freedman (1973b); the solid circle, the average given by Ponder (1948, p. 121).

Let us now turn our attention to the relation between Na' extrusion and the total pro-

tein contents of the red cell ghosts. In the AI hypothesis, water polarized in multilayers has reduced solubility for Na' and other large and complex molecules and ions. Model studies presented by Ling and Ochsenfeld (1983) show that typically, the apparent equilibrium distribution coefficient (or p-value), (which equals the true equilibrium distribution coefficient or q-value if the solute in question is exclusively water) decreases first rapidly then more slowly and eventually levels off with increasing polymer or protein concentration. To see if this pattern of Na' distribution also occurs in the red blood cell ghost, we calculated the p-values for Na^+ in the ghosts from their intracellular Na' concentrations given in Figure 3 and the external Na' concentrations in the final (18 hour) incubation solutions. The p-values thus calculated are plotted against the protein concentration of the ghosts and shown in Figure 6. In addition, Figure 6 includes the p-value of Na^+ in normal human erythrocytes at their normal protein content (34%). The p-value of 0.14 was calculated from the blood plasma Na' concentration of 138 mM (Edsall and Wyman, 1958, p. 18) and red cell Na^+ concentration of 19 mmoles/liter cell water (Bonting, 1970, p. 258). Virtually the same value can be calculated from the red cell Na' concentration (20 mM) given by Ponder (1948, p. 121). The best fitting curve obtained

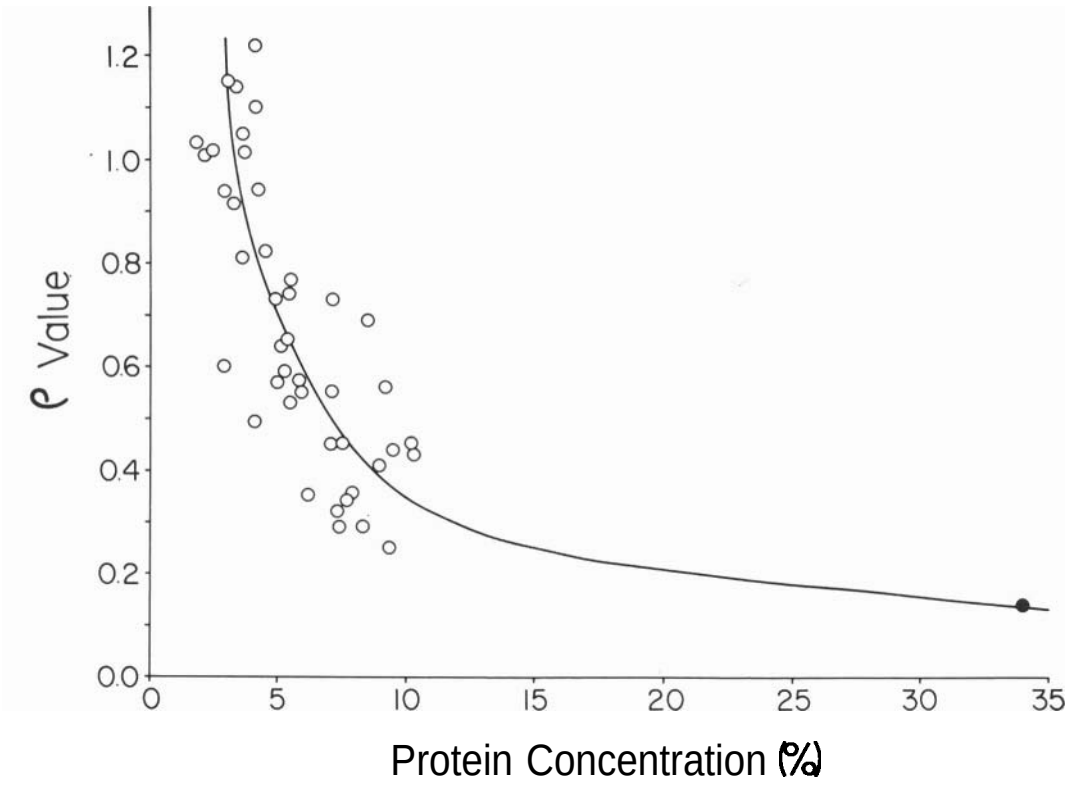

FIGURE 6. The apparent equilibrium distribution coefficient or p-value of Na^+ in red cell ghosts and of normal red blood cells plotted against the protein concentration of the ghosts or red cells. p-values for red cell ghosts were calculated from Na^+ contents of the ghosts and Na^+ concentrations in the incubation solutions after 18 hours of incubation and were derived from the same experiments providing the data of Figures 3 and 4. Na^+ concentration in normal human red blood cells, 19 mmoles/kilogram/fresh cells, and in blood plasma, 138 mM, were taken from Freedman (1973b).

by visual inspection does seem qualitatively at least to fulfill the expectation based on model studies. An exception is that at protein contents below 5%, the p-value actually exceeded unity. In terms of the AI hypothesis, this p-value above unity could only reflect Na' adsorption. There is not enough information to assess how much of the Na' observed is adsorbed but recent NMR studies suggest that up to 30% of human red cell Na^+ is bound (Gupta et al, 1982). However, it is quite clear that whereas the gain of K^+ with the increase of protein concentration is steadily rectilinear over the entire range of protein concentration up to that of normal red blood cells; the p-value drop for Na^+ is rectilinear only at protein concentrations below 10%.

The sharp decrease of p-value for Na' at low protein concentration suggests that the bulk of ghost water is effectively polarized in multilayers by only a small part of the protein (~) present in the cell. This efficiency is in harmony with the view that matrix protein(s) existing at a relatively low concentration provides the extended polypeptide chains needed for the polarization. One class of proteins that is ubiquitous enough to assume the role of the postulated matrix proteins is the cytoskeletal proteins, including actin, known to be present in the red cell ghosts (Tilney and Detmar, 1975). However, to serve as matrix protein, actin cannot exist in the filamentous form in which the backbone NHCO groups are locked in intramacromolecular H bonds. Rather it must be in a more finely dispersed and perhaps monomeric form (see Ling, 1984).

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