Effects of Ionic Strength on the Regulation of Na/H Exchange and K-Cl Cotransport in Dog Red Blood Cells

JOHN C. PARKER,*† PHILIP B. DUNHAM,*‡ and ALLEN P. MINTON§

From the *Department of Medicine, University of North Carolina, Chapel Hill, North Carolina 27599; ‡Department of Biology, Syracuse University, Syracuse, New York 13244-1220; and §Section on Physical Biochemistry, Laboratory of Biochemical Pharmacology, National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, Maryland 20892

ABSTRACT Dog red cell membranes contain two distinct volume-sensitive transporters: swelling-activated K-Cl cotransport and shrinkage-activated Na/H exchange. Cells were prepared with intracellular salt concentration and weight percentage of cell water (%cw) varied independently by transient permeabilization of the cell membrane to cations. The dependence of transporter-mediated Na and K influxes upon %cw and upon extracellular salt concentration (c_{ext}) was measured in cells so prepared. It was found that the critical value of %cw at which transporters are activated, called the set point, is similar for the two transporters, and that the set points for the two transporters decrease similarly with increasing extracellular salt concentration. These findings suggest a common mechanism of regulation of these two transporters.

Cellular Na, K, and Cl concentrations were measured as functions of %cw and c_{ext}. Using these data together with data from the literature for other solute concentrations, empirical expressions were developed to describe the dependence of the intracellular concentrations of all significant small molecule electrolytes, and therefore the intracellular ionic strength, upon %cw and c_{ext}.

A mechanistic model for the dependence of the set point of an individual transporter upon intracellular ionic strength is proposed. According to this model, the set point represents a critical extent of association between the transporter and a postulated soluble regulatory protein, called regulator. Model functions are presented for the calculation of the thermodynamic activity of regulator, and hence extent of regulator-transporter association, as a function of total intracellular protein concentration (or %cw) and ionic strength. The experimentally observed dependence of set point %cw on c_{ext} are simulated using these functions and the empirical expressions described above, together with reasonable but not uniquely determined values of model parameters.

†Deceased.

Address correspondence to P.B. Dunham, Biological Research Laboratory, Syracuse University, 130 College Place, Syracuse, NY 13244-1220.

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INTRODUCTION

Many animal cell types respond to changes in their fluid volume by activating ion transporters. The resultant salt fluxes and osmotically obliged movements of water serve to regulate cell volume (Sarkadi and Parker, 1991). Dog red cells have been useful in studying this phenomenon for several reasons. When exposed to anisotonic media, they swell or shrink within tenths of seconds, as water equilibrates across the plasma membrane. Within 1–10 min, membrane transporters promoting Na/H exchange are activated upon cell shrinkage, and membrane transporters promoting K-Cl cotransport are activated upon swelling (Parker, Colclasure, and McManus, 1991). Because these transporters are passive, and because cytoplasmic cation concentrations in dog red cells are so similar to those in plasma, there is little driving force for net transfer of Na or K across the membrane (Parker, 1977). The cells therefore remain very near steady state despite large increases in unidirectional transmembrane ion fluxes. It is thus possible to study the effects of cell volume on multiple cation transport pathways in a preparation of dog red cells by measuring the rate of uptake of radiolabeled ions as a function of cell water content under near steady state conditions.

In a typical plot of transporter-mediated Na or K flux vs cell water content, there is a region in which the slope of the plot changes abruptly. This region, the threshold for the activation of the flux as a function of cell volume, is termed the "set point." On one side of the set point, there is no discernible influence of water content on ion flux. On the other side of the set point, the flux increases strongly and monotonically with increasing difference between the set points and the actual cell volumes. The set point thus reflects a sharp maximum in the second derivative of the plotted function.

Previous work (Parker, McManus, Starke, and Gitelman, 1990) had shown two features of the set point for dog red cells. First, under a given set of experimental conditions, the set points for swelling-induced K-Cl cotransport and shrinkage-induced Na/H exchange were similar and possibly identical to within experimental precision. Second, the set points for these two distinct transporters moved in the same direction, and by roughly the same amount, in response to a number of experimental perturbations, including the introduction into the cell of urea and thiocyanate, consistent with the view that the two transporters are under the coordinated control of a single volume-regulatory signaling mechanism (Parker et al., 1990), as suggested earlier for duck red cells (Haas and McManus, 1985). See Motais, Guizoarn, and Garcia-Romeu (1991) for a related study in trout red cells.

We previously proposed (Minton, Colclasure, and Parker, 1992; Minton, 1994) that regulation of volume-sensitive ion transporters could be attributed to reversible association of the membrane-bound transporter with a soluble regulatory protein; the degree of activation of the transporter was a function of amount of regulatory protein(s) bound to the transporter at steady state. This proposal derives from work by Jennings and collaborators (Jennings and Al-Rohil, 1990; Starke and Jennings, 1993; see also Dunham, Klimczak, and Logue, 1993) indicating that volume-sensitive K-Cl cotransport in rabbit red cells is regulated by enzyme-catalyzed phosphorylation and dephosphorylation reactions. A protein phosphatase activates K-Cl cotransport by dephosphorylation of the transporter (or perhaps an associated regulatory

protein), and a kinase inhibits this transport pathway by promoting phosphorylation. It was proposed that only the kinase (and not the phosphatase) is volume sensitive; it is inhibited by cell swelling and activated by shrinkage.

In the simplest scheme, one soluble regulator, a volume-sensitive kinase, would inhibit K-Cl cotransport and stimulate Na/H exchange by binding to the transporters. However, the evidence for common volume-sensitive kinases(s) regulating the two transporters is not compelling, and a more general scheme for regulators binding with the transporters must be considered. So long as the regulator is a protein with mass comparable to or greater that that of hemoglobin, its tendency to bind to a regulatory site on a membrane-bound transporter will be extremely sensitive to changes in total intracellular protein, as measured by cell water content. For example, a 30% reduction in cellular volume might increase binding of regulator by as much as a factor of ten (Minton et al., 1992; Minton, 1994). Effects such as this are due to the large fraction of cellular volume excluded by hemoglobin to all macromolecules in the red cell, of low as well as high concentrations; these effects are general features of the equilibrium behavior of all concentrated macromolecular solutions (for a recent review, see Zimmerman and Minton, 1993).

If there is a soluble regulator that catalyzes a transporter-inhibiting reaction, there must also be a second regulator that catalyzes a transporter-activating reaction. This holds for the Jennings scheme: an inhibitory kinase which is volume sensitive, and a volume-insensitive stimulatory phosphatase. In our scheme, if there are pairs of regulators with opposite actions, one of the pair must be relatively insensitive to changes in concentration of the soluble proteins by virtue of high affinity binding to the transporters or some other type of compartmentation (Minton et al., 1992; Minton, 1994).

In the present work, we demonstrate that the alteration of intracellular ionic strength produces shifts in set points that are common to both types of transporters. To account for the direction and magnitude of these shifts, we propose an extension to a model for volume-sensitive activation of ion transporters earlier presented by us (Minton et al., 1992, Minton, 1994). According to this model, the high sensitivity of transporter-regulated ion flux to small changes in intracellular water content is postulated to reflect the strong dependence of the thermodynamic activity of intracellular proteins upon total protein concentration, or macromolecular crowding, which in turn strongly affects reversible associations between putative soluble regulatory proteins and membrane-bound ion transporters and the consequent level of transporter activity. In our extended model, the set point corresponds to a poising of the interaction between a membrane ion transporter and a single intracellular macromolecular regulator species at a critical soluble protein concentration and %cell water (%cw). The %cw of the set point in turn reflects a critical value of the thermodynamic activity of unbound regulator. When the thermodynamic activity coefficient of regulator is altered by some change in cellular contents, the set point changes to the new concentration of regulator corresponding to the original thermodynamic activity of regulator, and therefore, the original extent of interaction between regulator and transporter.

In the following section, we describe the experimental methods used to modify the water and salt content of dog red cells at steady state, and to measure the

transporter-regulated ion flux in these cells. The extended model is then introduced and methods of calculation described. Finally, the data obtained in the present study are presented and analyzed in the context of the model.

MATERIALS AND METHODS

Alteration of Cellular Water and Salt Content

Blood was obtained from the antecubital veins of mongrel dogs and centrifuged within 30 min. Plasma, white cells, and platelets were discarded and the cells washed three times by centrifugation and resuspension in a solution containing 140 mM NaCl, 4 mM KCl, 10 mM HEPES, and 5 mM glucose; in all experiments, the pH of all solutions was adjusted to 7.4 at 37°C with Tris base.

Mammalian red blood cell membranes have unusually low permeabilities to Na and K. The permeability to Na and K of a few types of cell membranes, including of dog red blood cells, is increased dramatically and reversibly by exposure to solutions containing ATP (Hempling, Stewart, and Gasic, 1969; Parker and Snow, 1972; Parker, 1977). Advantage was taken of this property of dog cells to modify their Na contents. Cells were washed twice in ATP-containing media which contained NaCl and sucrose concentrations varied reciprocally. All media had equal total solute concentrations (in millimolar): [NaCl] = (150 − [sucrose]/2) plus other constitutents, 1 mM ATP, 4 mM KCl, 10 mM HEPES, 5 mM glucose, pH 7.4. Cells were suspended in these media at a cell/medium volume ratio of ~1:10 and incubated in a 37°C shaker bath for 2 h. During this incubation, the cells lose or gain NaCl and water as a function of the sucrose concentration of their suspending medium. For example, cells lose NaCl and water in high sucrose, low Na media. After incubation, each lot of cells was centrifuged, and washed in a medium containing 140 mM NaCl plus KCl, glucose, buffers and pH as above. $MgSO_4$ was added to a final concentration of 0.5 mM to ensure reversal of the permeabilization by ATP (Parker and Snow, 1972). Each lot of cells was then washed twice with the medium to be used for influx determinations. Media with five different total salt concentrations were employed. All had KCl, glucose, buffers, and pH 7.4 as above plus NaCl at 75, 100, 140, or 200 mM. In some instances, salts of *N*-methyl-D-glucamine (NMDG), in inert substitute for Na, were used. NMDG Cl or NMDG NO_3 were substituted for NaCl to keep [Na] or [NaCl] constant. All media contained at least 75 mM NaCl. These substitutions for NaCl had no effect on the determinations of set points of the transporters.

The volume of the cells in these final media was a function of the Na concentration in the preincubation medium (the lower the Na concentration, the lower the salt content of the cells), and of the salt concentration in the final suspending medium. Therefore, in each experiment, the desired range of cell volumes in the final solutions determined the choice of NaCl and sucrose concentrations used in the ATP incubation. When cells containing different amounts of solute are suspended in the same medium, aliquots of cells of different volumes in a common medium are obtained. This alternative to varying the tonicity of the medium to vary cell volume has been used by others (e.g., Adragna and Tosteson, 1984).

Unidirectional Influxes

Cells were suspended in the flux media (cell/medium volume = 1/10), warmed to 37°C in a shaker bath for 5 min, and ^{22}Na or ^{86}Rb added to each suspension at a final concentration of 0.5–1.0 μCi (1–2 × 10^6 dpm)/ml. Previous work had demonstrated that entry of ^{22}Na and ^{86}Rb in both shrunken and swollen dog red cells under these conditions is linear with time over 40 min (Parker et al., 1990). Therefore, the influx was stopped after 30 min by centrifuging each suspension at 4°C. Cells were processed for counting by washing them twice with ice-cold,

isotope free flux media. A measured weight of cells was extracted with perchloric acid, and appropriate dilutions of the extract either counted directly for gamma radiation (^{22}Na) or added to scintillation fluid for beta counting (^{86}Rb). Unidirectional influxes were calculated as described earlier (Parker et al., 1990) and expressed as mmol/(kg dry wt × h).

Estimation of Set Points

Set point is defined phenomenologically as the threshold %cw for activation of transport as %cw is changed. There is a monotonic but highly nonlinear relationship between K or Na influx and %cw, manifested as a rapid change of slope from near-zero to a high-limiting value over a small range of %cw. The set point is assigned to the value of %cw corresponding to the intersection between a tangent to the region of limiting high slope and a tangent to the region of limiting low (near-zero) slope. In experiments with two to three estimates of flux in both the near-zero slope and high-slope regions of the curve, set points could be assigned fairly precisely to a narrow range of %cw. In a number of the experiments, there were insufficient numbers of estimates of influx in the zero-slope region to make a precise estimate of the intersection. To estimate set points in these experiments, we relied on the fairly constant flux in the near-zero slope region among experiments on both Na and K fluxes. For each experiment relating flux to %cw, a range of values of %cw for the set point, with upper and lower bounds, was estimated by eye. The extent of uncertainty of these estimates varied among experiments. For each data set, i.e., at a particular external salt concentration, means were obtained for upper and lower bounds of the set points (denoted <upper bound> and <lower bound>), and σ's for the upper and lower bounds were calculated. Then, $2\sigma_{\text{upper bound}}$ was added to mean upper bound and $2\sigma_{\text{lower bound}}$ was added to mean lower bound, giving estimates of 95% confidence regions. For several of the data sets, only one estimate of upper bound or lower bound (or both) was possible, and σ could not be determined. The method for displaying these uncertain estimates of bounds of the set points will be explained with the presentation of the data.

Determination of Cell Water and Dry Weight

After cells had been equilibrated with media to be used for influx measurements, a portion of each suspension was centrifuged at 40,000 *g*. The cell pellet was weighed (giving wet wt + dry wt), oven dried at 90° C for 30 min, placed in a microwave oven on the highest setting for 10 min, and weighed again (giving dry weight). A 1.7% correction was made for trapped medium in the packed cell pellets (Parker, 1971).

Measurement of the Intracellular Na, K, and Cl

Measurements were made on cells with ion concentrations and %cw's varied independently as described above. 16 sets of measurements were made on cells in media of five different compositions (three sets each in four of the media, four in the fifth). All contained KCl, glucose, buffers and pH 7.4 as above. In addition they contained: 75 mM NaCl, 100 mM NaCl, 100 mM NaCl + 40 mM NMDG Cl, 100 mM NaCl + 75 mM NMDG Cl, or 100 mM NaCl + 100 mM NMDG Cl. In each set of measurements in each of these media, cells were prepared at four different %cw's.

Na and K concentrations were measured on lysates of the cell samples by flame photometry (Instrumentation Laboratory model 943) using Cs as internal standard. Cl concentrations were measured by equilibrating cells 15 min with media containing ^{36}Cl and determining radioactivities of samples of cell lysates (extracted with perchloric acid) and of media by liquid scintillation counting. Intracellular Cl concentrations were calculated using the specific activities of ^{36}Cl in the media and the radioactivities of the lysates.

The ion concentrations are expressed in mmol/liter cell water. A trapped volume of medium of 1.7% in packed pellets of cells was assumed in the calculations. The calculated ion concentrations are insensitive to this assumption because extracellular and intracellular concentrations of none of the three ions were very different for any of the experimental conditions employed.

Model for the Effect of Small Molecule Solutes on the Set Points(s) of Volume-sensitive Ion Transporters

In the following section we present experimental evidence that increasing intracellular ionic strength shifts the set points of the K-Cl and Na/H transporters to higher cytoplasmic protein concentrations.

Based upon these observations, we propose the following hypothesis: the set point of a particular transporter represents a critical level of association between that transporter and the corresponding putative regulatory protein, which we will call regulator and denote by R. (The regulator may or may not be the same protein for the two transporters.) The level of association between regulator and transporter is governed by the thermodynamic association constant and the thermodynamic activity (as distinct from concentration) of free regulator. The basic assumption underlying the model developed below is that the set point represents a particular value of the thermodynamic activity (or, alternatively, the chemical potential) of free regulator that is fixed at constant temperature and pressure. This may be formally stated as:

$$a_R^{SP} \equiv a_R(\{c_{SM}^{SP(1)}\}, \{c_{MM}^{SP(1)}\}) = a_R(\{c_{SM}^{SP(2)}\}, \{c_{MM}^{SP(2)}\}) \tag{1}$$

where a_R denotes the thermodynamic activity of regulator, $\{c_{SM}^{SP}\}$ and $\{c_{MM}^{SP}\}$ denote respectively the set point concentrations of all small molecule and all macromolecular solutes, and the superscripts 1 and 2 denote the values of the corresponding variables at two different intracellular compositions. For example, conditions 1 and 2 might denote different intracellular volumes or concentrations of salt, urea, et cetera. It follows from Eq. 1 that any change in %cw at the set point brought about by a perturbation of intracellular composition derives from a corresponding change in the concentration of regulator at fixed activity, i.e., a change in the activity coefficient of regulator.

Free Energy Relations and Constituent Activity Coefficients

The various contributions to the thermodynamic activity of regulator may be readily discerned through analysis of the chemical potential of regulator, which may be written as a sum of differential free energy contributions. We assume that deviations from the ideal chemical potential of *R*, defined as

$$\mu_R^{ideal}(T, P, c_R) \equiv \mu_R^o(T, P) + RT \ln c_R \tag{2a}$$

may be attributed to interactions between R and small molecule solutes on the one hand, and between *R* and other macromolecular solutes on the other:

$$\begin{aligned}\mu_R(T, P, \{c_{SM}\}, \{c_{MM}\}) &= \mu_R^o(T, P) + RT \ln a_R(\{c_{SM}\}, \{c_{MM}\}) \\ &= \mu_R^{ideal} + \delta\mu_R^{SM}(\{c_{SM}\}) + \delta\mu_R^{MM}(\{c_{SM}\}, \{c_{MM}\}),\end{aligned} \tag{2b}$$

where μ_R^o denotes the standard state chemical potential, defined with respect to an ideal solution of R at *T*, *P*, and unit concentration of *R*, $\delta\mu_R^{SM}$ denotes the incremental free energy of interaction between dn_R moles of R and the small molecule solutes in the solution, and $\delta\mu_R^{MM}$ denotes the incremental free energy of interaction between dn_R moles of R and the macromo-

lecular solutes in the solution. It is assumed here that whereas small molecule solutes may modulate interactions between R and other macromolecules, macromolecular solutes do not affect the interaction between R and small solutes. We define the constituent activity coefficients γ_R^{SM} and γ_R^{MM} according to

$$\begin{aligned} \gamma_R^{SM} &\equiv \exp(\delta\mu_R^{SM}/RT) \\ \gamma_R^{MM} &\equiv \exp(\delta\mu_R^{MM}/RT). \end{aligned} \tag{3}$$

It follows from Eqs. 2 and 3 that

$$a_R(c_R, \{c_{MM}\}, \{c_{SM}\}) = \gamma_R^{MM}(\{c_{MM}\}, \{c_{SM}\}) \times \gamma_R^{SM}(\{c_{SM}\}) \times c_R. \tag{4}$$

Eqs. 1 and 4 may be combined to yield

$$\frac{c_R^{(2)}}{c_R^{(1)}} = \frac{\gamma_R^{MM}(\{c_{MM}^{(1)}\}, \{c_{SM}^{(1)}\})}{\gamma_R^{MM}(\{c_{MM}^{(2)}\}, \{c_{SM}^{(2)}\})} \times \frac{\gamma_R^{SM}(\{c_{SM}^{(1)}\})}{\gamma_R^{SM}(\{c_{SM}^{(2)}\})}. \tag{5a}$$

The absolute concentration of regulator is of course unknown, but because the plasma membrane is impermeable to all proteins,

$$\frac{c_{Hb}^{(2)}}{c_{Hb}^{(1)}} = \frac{c_R^{(2)}}{c_R^{(1)}}. \tag{5b}$$

The ratio of the final and initial values of hemoglobin concentration may be expressed as a function of the initial and final values of %cw (Appendix I).

Dependence of γ_R^{MM} upon $\{c_{SM}\}$ and $\{c_{MM}\}$

The intrinsic activity coefficient reflecting the interaction between R and all other macromolecules in solution may be written as an expansion in powers of the concentrations of macromolecular species:

$$\ln \gamma_R^{MM} = \sum_j B_{R,j}(\{c_{SM}\})c_j + \sum_{j,k} B_{R,j,k}(\{c_{SM}\})c_j c_k + \cdots \tag{6}$$

where the $B_{R,j}$, $B_{R,j,k}$, . . . , are functions of the potential of interaction between a molecule of R and one, two, . . . other macromolecules in a solution containing the concentration of small molecule solutes indicated by $\{c_{SM}\}$ (Hill, 1960). In a red blood cell, the concentration of hemoglobin is orders of magnitude higher than that of any other macromolecule (including R), and so Eq. 6 reduces to

$$\ln \gamma_R^{MM} = B_{R,Hb}(\{c_{SM}\})c_{Hb} + B_{R,Hb,Hb}(\{c_{SM}\})c_{Hb}^2 + \cdots \tag{7}$$

It will subsequently be shown that under the conditions of the experiments reported here, the net charge on hemoglobin is sufficiently small that electrostatic contributions to the interaction between hemoglobin and regulator are likely to be negligible relative to the contribution of volume exclusion deriving from the mutual impenetrability of two macromolecules. We shall therefore assume that the dependence of the various interaction coefficients $B_{i,j}$, $B_{i,j,k}$, . . . upon the concentration of small molecule electrolytes is negligible. Under such conditions one may usefully approximate compact globular proteins by equivalent hard spherical particles having dimensions close to those of the corresponding protein molecules (Ross and Minton, 1977; Minton, 1983). In addition, one may estimate the activity coefficient of each species of protein in a mixture of compact globular proteins using results obtained from the scaled particle theory of hard sphere mixtures (Lebowitz, Helfand, and Praestgaard, 1965; Chatelier and Minton,

1987). According to this theory, the macromolecular contribution to the activity coefficient of regulator may be expressed as

$$\ln \gamma_R^{MM}(r_R) = A_0(c_{Hb}, r_{Hb}) + A_1(c_{Hb}, r_{Hb})r_R + A_2(c_{Hb}, r_{Hb})r_R^2 + A_3(c_{Hb}, r_{Hb})r_R^3 \quad (8)$$

where r_R and r_{Hb} denote the radii of the equivalent hard spherical particles representing regulator and hemoglobin respectively, and the A_i are geometric functions describing volume exclusion properties of the effective hard spheres representing hemoglobin (Chatelier and Minton, 1987, Appendix).

Dependence of γ_R^{SM} upon $\{c_{SM}\}$

The value of $\delta\mu_R^{SM}$, and hence γ_R^{SM}, is determined by the nature of specific and nonspecific interactions between small molecule solutes and the regulator. In the absence of information about the chemical nature of the putative regulatory protein, we shall consider only nonspecific electrostatic interactions between small molecule electrolytes and regulator. For purposes of quantitation we shall employ the extended Debye-Hückel calculation of the electrostatic free energy of a globular protein in a salt solution, wherein the protein is modeled as a sphere with a net charge of Z dispersed on its surface (Tanford, 1961). This treatment leads to the expression

$$\ln \gamma_R^{SM} = -\frac{Z^2e^2}{2\epsilon kT} \times \frac{\kappa}{1 + \kappa a} \quad (9a)$$

where

$$\kappa = \left\{\frac{8\pi N_A e^2}{1000\epsilon kT}\right\}^{1/2} \times I^{1/2} \quad (9b)$$

and

$$I = \frac{1}{2}\sum_i c_i z_i^2. \quad (9c)$$

In Eqs. 9, a–c, e denotes the charge of the electron in cgs electrostatic units, ϵ the dielectric constant of solvent (taken to be equal to 80), k the Boltzmann constant, T the absolute temperature, κ the inverse Debye length, a the distance of closest approach of ions to the center of the spherical protein, N_A Avogadro's number, I the ionic strength, and c_i and z_i the molar concentration and charge of the ith species respectively.

RESULTS

Preparation of Cells with Varying Salt and Water Contents

Fig. 1 shows that cells can be made with varying cell water contents by preincubation in media containing ATP (which raises cation permeability) and varying NaCl and sucrose concentrations to increase or decrease the Na content of the cells. After removal of ATP to restore the initial cation permeability, the various aliquots of cells were suspended in media with either 100 mM NaCl or 100 mM NaCl + 75 mM NMDG NO_3 (both media also had 4 mM KCl and buffers as described in Methods). Water content of cells equilibrated in these media decreases approximately linearly with increasing concentration of the impermeant solute, sucrose, in the preincubation media. The higher the extracellular sucrose concentration during ATP perme-

abilization, the more Na was extracted from the cells and therefore, the lower their water content after reversal of the ATP permeabilization. Because the cells are in osmotic equilibrium with their suspending medium, their volume or water content in any given medium is a function of their solute content, mainly NaCl. For example, cells preincubated in an ATP-containing medium with 130 mM NaCl (+ sucrose) and then washed and suspended in 175 mM salt or 100 mM salt will equilibrate to cell volumes of ~62 and 70%cw, respectively. Likewise, cells preincubated in an ATP-containing medium with 90 and 130 mM NaCl (+ the appropriate sucrose concentrations) and then washed and suspended in 100 mM salt will equilibrate to volumes of 61 and 70%, respectively. Thus, varying the salt (and sucrose) concentrations of the preincubation media and the salt concentration of the final media allowed us to prepare cells with water content and salt concentration varied independently.

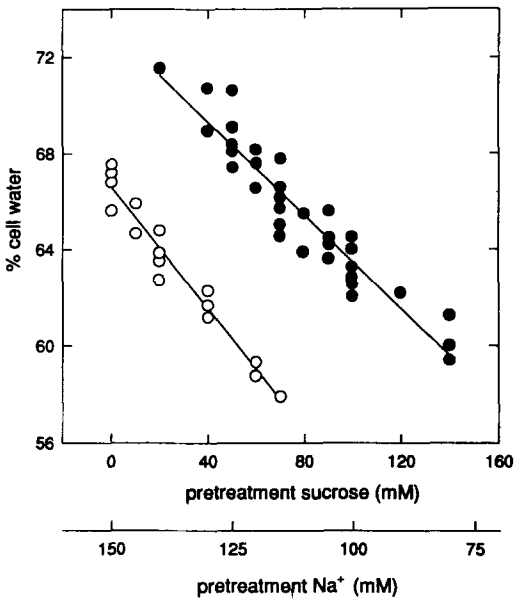

FIGURE 1. Cell water content as a function of sucrose and NaCl concentrations in preincubation media. The preincubation media contained sucrose and NaCl at the concentrations given on the abscissae. All media had equal total solute concentrations (see Materials and Methods). After 2 h preincubation, cells were washed once to free them of ATP with a medium containing 140 mM NaCl, 4 mM KCl, 10 mM HEPES, and 0.5 mM $MgSO_4$, pH 7.4. Before cell water measurements, cells were washed twice with either a 175-mM salt medium (100 mM NaCl + 75 mM NMDG NO_3) (*open symbols*) or a 100 mM NaCl medium (*filled symbols*). These media also contained 4 mM KCl, 5 mM glucose, and buffer, pH 7.4. Pooled results from 10 experiments are shown.

K and Na Influxes in Cells with Varying Water Contents

Fig. 2 shows results of a typical experiment on the dependence of unidirectional K and Na influxes on cell water content in two sets of cells in flux media with either 100 mM NaCl (*filled symbols*) or 100 mM NaCl + 75 mM NMDG NO_3 (*open symbols*), as in Fig. 1. At a particular water content, the cells in media with 175 mM salt contained higher salt concentrations than those in 100 mM salt (results shown below). For a given set of cells, increasing cell water markedly stimulated K influx and decreasing cell water markedly stimulated Na influx. Changes in cell water of ~3% activated the fluxes by >fivefold, confirming earlier results with dog red blood cells (Parker et al., 1990). We present evidence below that the volume-sensitive fluxes are entirely K-Cl cotransport or Na/H exchange, respectively.

For both K and Na fluxes, the set points for the volume-sensitive fluxes were different in cells incubated in media with different salt concentrations. Given data comparable to that shown in Fig. 2, one cannot precisely define a unique set point, but one can define a range of %cw encompassing the set point; the range is defined by an upper bound and a lower bound (see Materials and Methods). We made visual estimates of this range for each of the data sets, indicated in Fig. 2 as thick horizontal bars drawn between estimated lower and upper bounds. The set points for both Na and K fluxes in the high salt medium were ~62%cw, whereas in the low salt medium the set points for both fluxes were 65–66%cw. The differences in set points were not due to differences in extracellular Na or Cl concentrations because they were the

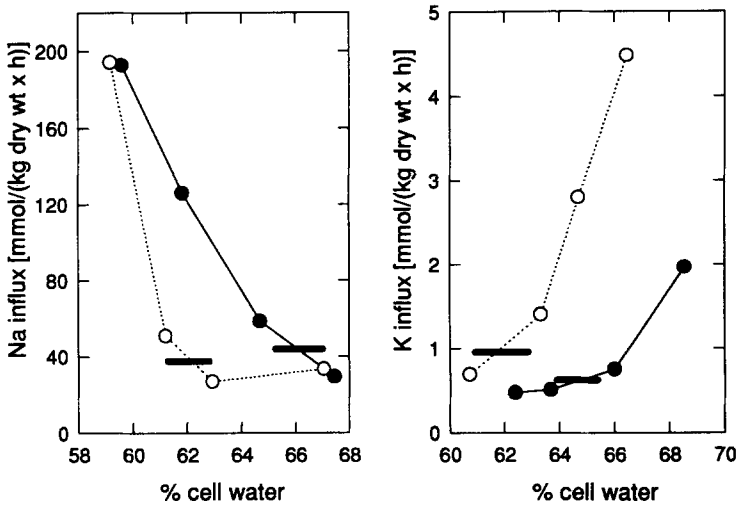

FIGURE 2. Unidirectional Na (*left*) and K (*right*) influxes as functions of cell water content. (*Filled symbols; solid lines*) Experiments in which all cells were suspended in a medium containing 100 mM NaCl and other constituents as given for Fig. 1. (*Open symbols; dashed lines*) Experiments in which all cells were suspended in a medium containing 100 mM NaCl + 75 mM NMDG NO_3 plus the other constituents. Cell water content had been varied as described in Materials and Methods by incubating cells in ATP-containing media for 2 h before the flux period. The heavy horizontal bars associated with the results of each experiment indicate the range of lower and upper bounds of the estimates of the four set points, two each for Na and K influxes (see text for details). Each panel represents a single experiment.

same in the two media. Furthermore, 175 mM NaCl gave essentially the same results as 100 mM NaCl + 75 mM NMDG NO_3 (results not shown).

The data shown in Fig. 3 indicate that the volume-sensitive fluxes measured in cells preincubated with ATP were mediated by the expected transporters. Shrinkage-activated Na flux was entirely inhibited by amiloride and is therefore Na/H exchange (Parker, Gitelman, and McManus, 1989). The swelling activated K flux was for the most part chloride dependent, consistent with it being K-Cl cotransport (Parker et al., 1990). The slight increase in the flux in Cl-free medium was seen consistently and may indicate that the substitute anion (in this case sulfamate) can be a low affinity

alternative substrate for the cotransporter. Similar results were obtained with NO_3 as the substitute anion. Chemical analysis of putative Cl-free media and cells from them gave Cl concentrations < 1 mM.

Summary of Estimates of Set Points

Experiments like those summarized in Fig. 2 were carried out a total of 12 times for K fluxes and 24 times for Na fluxes. In the experiments in Fig. 2, the salt concentrations in the final media (in addition to KCl and buffers) were 100 mM and 175 mM. In other experiments, media contained 75, 140, or 200 mM salt. The media contained at least 75 mM NaCl; in some instances the media were supplemented with NMDG Cl or NMDG NO_3 to keep [Na] or [NaCl] constant. As stated above, the results on set points were indistinguishable from those with all NaCl media.

In each experiment, fluxes were compared for two sets of cells in different salt solutions, each set with a range of cell waters. Estimates were made of set points in all

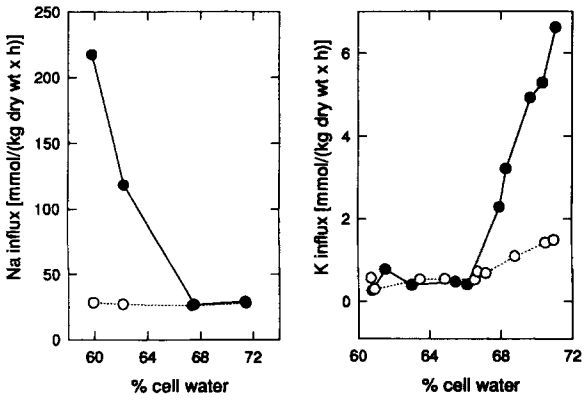

FIGURE 3. Unidirectional Na (*left*) and K (*right*) influxes as functions of cell water content. (*Filled symbols*) Experiments in which all cells were suspended in a medium containing 140 mM NaCl and KCl, glucose, and buffers as in Fig. 1. (*Open symbols, left*) Addition of 0.4 mM amiloride to the flux medium. (*Open symbols, right*) Substitution of sulfamate for chloride in the flux medium. Cell water content was varied by preincubation of cells with ATP and sucrose as described in Materials and Methods. The left and right show results of one and three experiments, respectively.

of these experiments as described in Materials and Methods, and the combined results are summarized in Fig. 4, *A* and *B*. The heavy vertical bar for a given external salt concentration indicates the 95% confidence region for the set point at that external salt concentration (see Materials and Methods for estimation of confidence regions). Uncertain 95% confidence limits are indicated by arrow points (see figure legend for explanation). Although the data are noisy and the confidence regions are broad, the general trend is clear; set points for both Na and K transporters are quite similar (if not identical) at all external salt concentrations, and the cell water content at a particular set point increases significantly as the external salt concentration decreases.

Intracellular Concentrations of Na and K

The sums of the intracellular concentrations of sodium and potassium, measured as described in Materials and Methods, are plotted as a function of %cw and external

salt concentration in Fig. 5 *A*. These data were fitted by the method of linear least squares by the empirical regression function:

$$c_{Na+K}(mM) = C_0 + C_1\ \%cw + C_2 c_{ext} \tag{10}$$

where c_{ext} denotes the total extracellular concentration of uniunivalent salt in millimolar units. The form of Eq. 10, which defines a planar surface, was suggested by the appearance of the plot of the data in Fig. 5 *A*. It should be noted that the concentration of intracellular salt is in units of millimoles per liter of cell water, i.e., the volume of intracellular hemoglobin is not included. The standard deviation of the residuals is 5.8 mM, generally within the scatter of experimental data, and no gross systematic deviations are evident. We therefore accept the best-fit function as a description of the combined data to within experimental uncertainty.

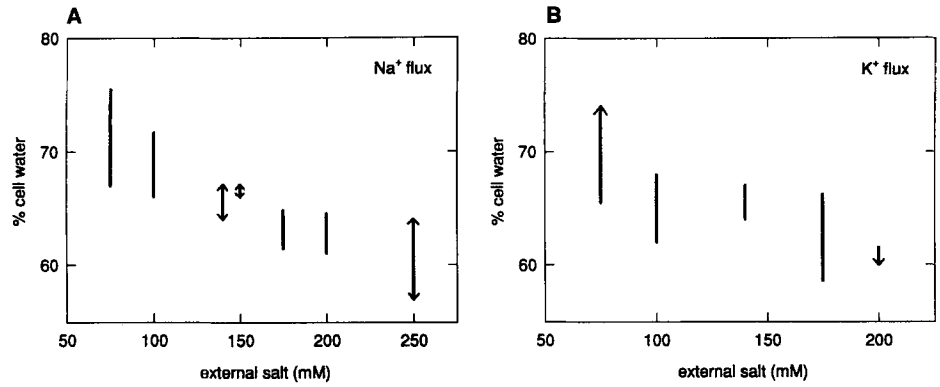

FIGURE 4. Summary of estimates for set points as functions of salt concentration in the flux media (external salt). (*A*) 24 experiments on the Na/H exchanger. (*B*) 12 experiments on the K-Cl cotransporter. Each vertical bar represents a ~95% confidence region for the estimates of set points as described in Materials and Methods. An arrow point indicates that only one estimate of the corresponding bound was obtained; although a 95% confidence limit could not be calculated, it must be at least as high (in the case of an upper bound) or at least as low (in the case of a lower bound) as the value indicated by the arrow point.

Intracellular Concentrations of Cl

The Cl concentrations, measured as described in Materials and Methods and plotted in Fig. 5 *B*, were fitted by the method of linear least squares by the following empirical regression function

$$c_{Cl}(mM) = D_0 + D_1\ \%cw + D_2 c_{ext} \tag{11}$$

where c_{ext} denotes the total extracellular concentration of uniunivalent salt in millimolar units. The standard deviation of residuals is 9.6 mM, somewhat larger than that obtained from fitting a similar function to the intracellular cation data. This difference seems to derive from systematic deviations in one subset of the data, namely the subset obtained at c_{ext} = 179 mM, which appears to have a marginally

larger dependence of c_{CL} upon %cw than predicted by the best fit of the model function. Because data obtained at the other four extracellular salt concentrations seem to be mutually consistent with each other and with the model, we accept the best fit function as a description of the combined data to within experimental uncertainty.

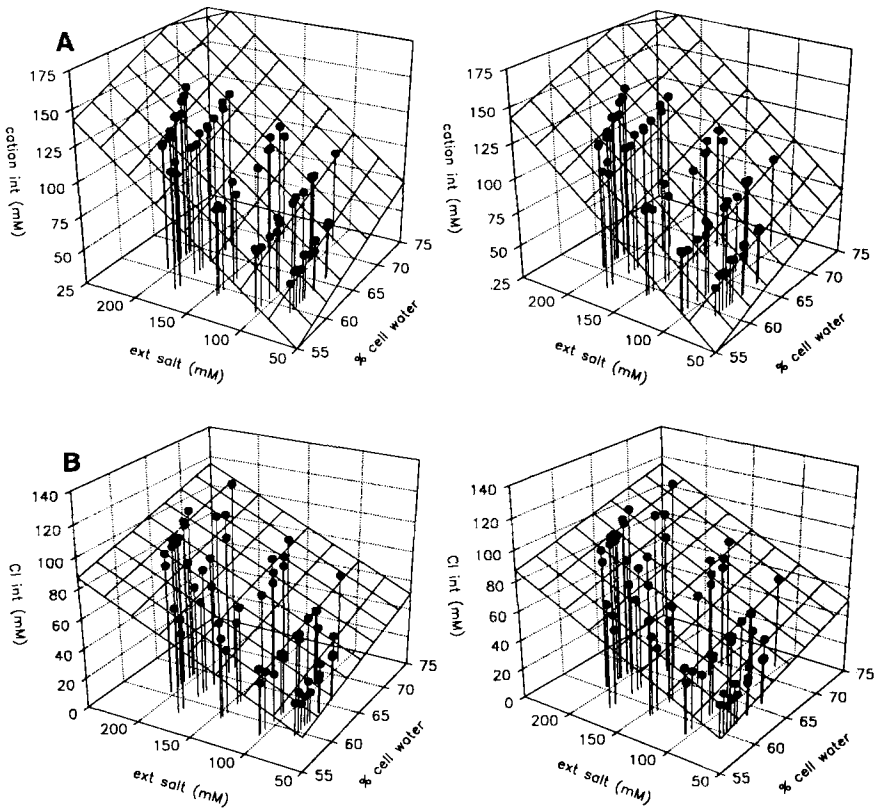

FIGURE 5. Concentrations of intracellular ions plotted vs external salt concentration and %cw (*stereo views*). Points represent experimental data, and the mesh surfaces represent the best fits of Eqs. 10 or 11 to the corresponding data sets. (*A*) Na + K concentrations (n = 63). The best-fit surface was calculated using Eq. 10 with $C_o = -135.87$; $C_1 = 2.3857$; $C_2 = 0.58869$. (*B*) Cl concentrations (n = 63). The best-fit surface was calculated using Eq. 11 with $D_0 = -176.43$; $D_1 = 2.5124$; $D_2 = 0.57353$.

MODEL CALCULATIONS

Hemoglobin Charge

The net charge on intracellular hemoglobin in dog red blood cells was calculated as a function of %cw and external salt concentration via the condition of electroneutrality, as described in Appendix III. The results are plotted in Fig. 6. The net charge per hemoglobin molecule has essentially no dependence upon external salt concentration at constant cell water, and is smaller in magnitude than that reported for human

hemoglobin, −5.6, at comparable pH (Bisognano, Dix, Pratap, Novak, and Freedman, 1993). Though invariant with external salt concentration for fixed cell water, the calculated hemoglobin charge in dog red cells varied with cell water, becoming slightly more negative with decreased %cw. This small apparent dependence of hemoglobin charge on %cw is within the uncertainty of the calculation, and may not be real. The salient feature of the results in Fig. 6 is that the charge on dog hemoglobin is low and changes little with large changes in cell volume, hemoglobin concentration, and salt concentration. As stated above, the small net charge of hemoglobin means that its electrostatic interactions with regulator are likely to be negligible relative to volume exclusion effects.

Earlier work (Ross and Minton, 1977; Ross, Briehl, and Minton, 1978) established that under conditions similar to those in the present study, departures from thermodynamic ideality in concentrated solutions of hemoglobin may be fairly accurately calculated if one represents the hemoglobin molecule as an effective hard

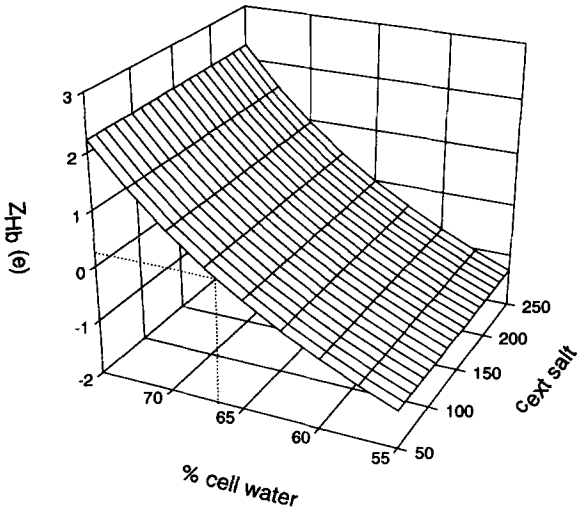

FIGURE 6. Net charge on hemoglobin as a function of %cw and external salt concentration, calculated using equation (A3.2). Under physiological conditions dotted lines represent (67 %cw) the net charge on hemoglobin is close to zero.

spherical particle with r_{Hb} = 2.9 nm; we shall use this representation in the present work.

Dependence of Set Point upon External Salt Concentration

Recognizing that γ_R^{MM} may be expressed as a function of hemoglobin concentration only (see above), Eqs. 5a and 5b may be combined and rewritten as

$$c_{Hb}^{(2)} \times \gamma_R^{MM}(c_{Hb}^{(2)}) \times \gamma_R^{SM}(\{c_{SM}^{(2)}\}) = c_{Hb}^{(1)} \times \gamma_R^{MM}(c_{Hb}^{(1)}) \times \gamma_R^{SM}(\{c_{SM}^{(1)}\}). \quad (12)$$

Utilizing this relation, the curves plotted in Fig. 7, *A* and *B* (superimposed on the results in Figs. 4, *A* and *B*), were calculated as follows.

First, the values of the following input parameters are specified: (*a*) an arbitrarily selected reference concentration of external salt ($c_{ext}^{(1)}$); (*b*) the set point cell water

content at the reference salt concentration (%cw$^{(1)}$); (*c*) the effective hard sphere radius of the putative regulatory protein (r_R); and (*d*) the net charge of the putative regulatory protein (Z_R). Given %cw$^{(1)}$ and $c_{ext}^{(1)}$, $c_{Hb}^{(1)}$ and the set of $\{c_{SM}^{(1)}\}$ are calculated using Eqs. A1.1 and A2.1–A2.3. Then, γ_R^{MM} ($c_{Hb}^{(1)}$) is calculated using Eq. 8 and γ_R^{MM} ($\{c_{SM}^{(1)}\}$) calculated using Eq. 9a, with the value of a taken to be equal to r_R. Then the value of the expression on the right hand side of Eq. 12 is calculated. Because the set point is assumed to reflect a condition of constant thermodynamic activity of regulator, the value of the right hand side is held constant for the set of input parameters introduced above. The expression on the left hand side of Eq. 12 may be similarly calculated as a function of of %cw$^{(2)}$ and $c_{ext}^{(2)}$, permitting the numeric solution of Eq. 12 for %cw$^{(2)}$ as an implicit function of $c_{ext}^{(2)}$. Each of the curves plotted in Fig. 7, *A* and *B*, represents %cw$^{(2)}$ calculated as a function of $c_{ext}^{(2)}$ for a single set of input parameters specified in the figure caption.

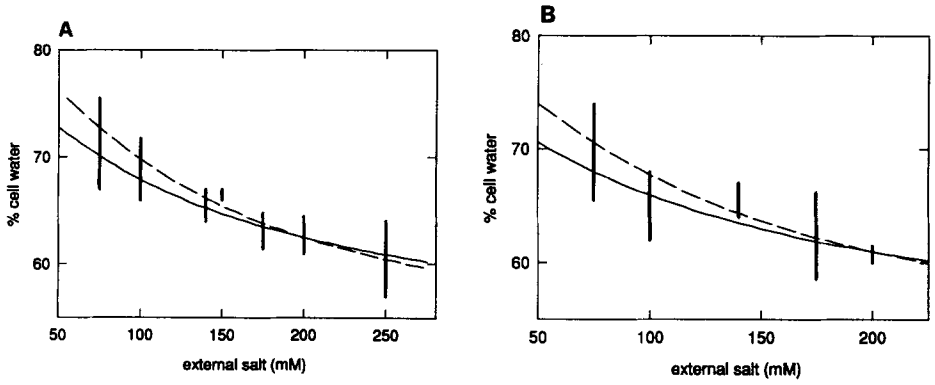

FIGURE 7. Model calculations of %cw as a function of external salt concentration. Curves are calculated as described in the text, based on the assumption of constant thermodynamic activity of regulator (Eq 1), with r_R = a = 20 × 10^{-8} cm, and Z_R = 14 (*solid curve*) or 16 (*dashed curve*). (*A*) Curves calculated for 61% cell water at 200 mM external salt, overlaid on cumulative set point data for K flux. (*B*) Curves calculated for 62.5% cell water at 200 mM external salt, overlaid on cumulative set point data for Na flux.

DISCUSSION

Red cells are so permeable to water that they are essentially at osmotic equilibrium with their surroundings in the time scale of our experiments. Because of this, one can manipulate cell water content by changing either the tonicity of the bathing medium or the solute content of the cells. Most investigations of cell volume regulation have used the device of changing medium tonicity to vary cell volume. Despite the ease of this approach, there is the disadvantage that responses to this type of maneuver could be due either to changes in cell volume or to changes in the concentration of solutes in the media.

In the present study we pretreated the cells to alter their solute content by permeabilizing them reversibly with extracellular ATP and then adding or subtracting salt from them. By suspending cells with different amounts of solute in a common

medium, we varied cell volume while maintaining medium tonicity constant. The relationships between cell water content, Na flux, and K flux were similar to those found in experiments with varying the tonicity of the media: shrinkage activated amiloride-sensitive Na flux and swelling activated chloride-dependent K flux. Thus, the responses of the Na/H exchanger and the K-Cl cotransporter to shrinkage and swelling are not due to changes in tonicity but are related to cell volume or water content.

Next we compared the relationships between transport and cell volume in cells with comparable volumes suspended in media of differing salt concentrations. We made several batches of cells that would assume water contents ranging from 60 to 72% cell water when suspended in a 100 mM salt medium (Fig. 1). A second series was prepared that had a range of 59–68% cell water when suspended in a 175 mM salt medium (Fig. 1). We observed a decrease in the set point of cells suspended in 175 mM as compared with 100 mM salt. The change affected both shrinkage-induced Na/H exchange and swelling-induced K-Cl cotransport. Both set points were shifted in the same direction, to lower cell volumes at higher salt concentration, and to approximately the same degree (Fig. 2). Extensive additional experiments, the results of which are summarized in Fig. 4, confirmed and extended these initial findings. These results suggested that either total solute concentration or ionic strength could be a modulator of the volume response in dog red cells. A point in favor of solute concentration was the observation that urea, which equilibrates rapidly across the red cell membrane and does not influence cell water content, causes a similar, downward set point shift in both Na/H exchange and K-Cl cotransport (Parker, 1993*a*). However, methanol, which like urea changes the solute concentration but not the volume of the cells, has no effect of the set point of the cells in concentrations up to 400 mM (Parker, 1993*b*). Therefore, the effect of intracellular urea on set point is most likely due to specific interactions between urea and intracellular proteins rather than a generalized consequence of increased small molecule solute concentration. In like manner, the dependence of set point on intracellular salt concentration reported here is most likely due to nonspecific electrostatic interactions between small electrolytes and intracellular proteins, including the putative regulators for the volume-sensitive ion transporters.

To express the chemical potential of the regulator R as a function of the intracellular concentrations of R, hemoglobin, and small elecrolytes, Eqs. 2–4 together with the various model assumptions presented earlier were combined to yield the following expression:

$$\mu_R = \mu_R^o + RT \ln c_R + RT \ln \gamma_R^{MM}(c_{Hb}) + RT \ln \gamma_R^{SM}(\{c_{SM}\}) \quad (13)$$

The concentration of regulator is unknown but is proportional to that of hemoglobin ($c_R = \beta c_{Hb}$). Therefore Eq. 13 may be rewritten as

$$\mu_R = \mu_R^* + RT \ln a^*(c_{Hb}) + RT \ln \gamma_R^{SM}(\{c_{SM}\}) \quad (14)$$

where μ_R^* ($\equiv \mu_R^o + RT \ln \beta$) is independent of salt and hemoglobin concentration, and hence constant at constant temperature and pressure, a^* ($\equiv \gamma_R^{MM}(c_{Hb}) \times c_{Hb}$) is independent of salt concentration, and γ_R^{SM} is independent of hemoglobin concentra-

tion. Note that a* is not the thermodynamic activity of either R or hemoglobin; it is, however, a defined (and calculable) function of hemoglobin concentration.

The dependence of the set point hemoglobin concentration (or %cw) upon salt concentration may be visualized in the following manner. Because $\mu_R = \mu_R^o + RT \ln a_R$, Eq. 14 may be used to calculate a_R as a function of the concentration of hemoglobin and small molecule electrolytes. The solid curve in Fig. 8 represents the dependence of a_R upon c_{Hb}, calculated using Eq. 14, for a particular set of intracellular electrolyte concentrations denoted by $\{c_{SM}^{(1)}\}$. Let the set point at this salt concentration correspond to an intracellular hemoglobin concentration of $c_{Hb}^{SP(1)}$. According to the model presented here, this means that at the cytosolic composition defined by $c_{Hb}^{SP(1)}$ and $\{c_{SM}^{(1)}\}$, the value of a_R is poised at a critical value such that a small change in one direction will have a large, unequivocally detectable effect on the ion flux, while a small change in the opposite direction will have a small, experimentally indetectable effect. We thus call this particular value of activity the **set point activity**

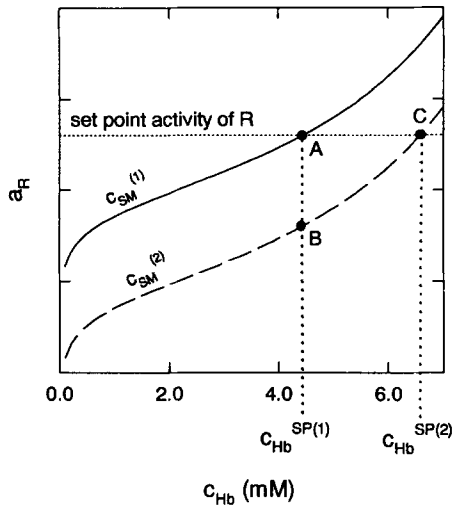

FIGURE 8. a_R, the thermodynamic activity of regulator, plotted as a function of c_{Hb} for two different intracellular salt concentrations, $c_{SM}^{(1)}$ (*solid curve*), and $c_{SM}^{(2)}$ (*dashed curve*), where $c_{SM}^{(2)} > c_{SM}^{(1)}$. $r_R = 25 \times 10^{-8}$ cm. The particular value of the chemical potential of free regulator associated with the poising of interactions between regulator and ion transporter characteristic of the set point is indicated by the horizontal dotted line. Hence, the set point for a given salt concentration is found at the particular hemoglobin concentration c_{Hb}^{SP} (and the corresponding value of %cw) defined by the intersection between the corresponding curve and the horizontal dotted line. (*A*, *B*, and *C*) Explained in the text.

of **R,** or a_R^{SP}; it is indicated by a horizontal dotted line in Fig. 8. The intersection is indicated by a solid dot marked *A*.

Let us now change the cytosolic composition of electrolytes to $\{c_{SM}^{(2)}\}$, such that at the new composition the intracellular ionic strength is greater than at $\{c_{SM}^{(1)}\}$. It follows from Eq. 14 that the dependence of a_R upon c_{Hb} at $\{c_{SM}^{(2)}\}$ will be identical to that at $\{c_{SM}^{(1)}\}$ except for a constant vertical offset equal to $RT \ln [\gamma_R^{SM}(\{cSM(2)\})/\gamma_R^{SM}(\{c_{SM}^{(1)}\})]$, which is negative in the present case, as γ_R^{SM} decreases with increasing ionic strength, i.e.,

$$a_R(c_{Hb}^{SP(1)}, \{c_{SM}^{(2)}\}) = a_R^{SP} + RT \ln [\gamma_R^{SM}(\{c_{SM}^{(2)}\})/\gamma_R^{SM}(\{c_{SM}^{(1)}\})] \quad (15)$$

The dependence of a_R upon c_{Hb} at $\{c_{SM}^{(2)}\}$ is shown as a dashed curve in Fig. 8, and the value of a_R after the change of intracellular salt concentration at constant hemoglobin concentration indicated by a solid dot marked *B*. Because the thermodynamic activity

of R has changed, so has the sensitivity of the ion transporter to changes in the activity (or concentration) of R. The critical poising of the interaction between R and ion transporter that is characteristic of the set point will only be restored when the hemoglobin concentration changes (at constant intracellular salt) to a new value such that $a_R = a_R^{SP}$. This point is indicated in Fig. 8 by a solid dot marked *C*. According to the model, the hemoglobin concentration at this point will be the new (higher) set point hemoglobin concentration, $c_{Hb}^{SP(2)}$, corresponding to a new (lower) set point %cw.

The quantitative model presented here thus provides a plausible explanation of the observed dependence of set point cell water content on intracellular small electrolyte concentration, i.e., ionic strength. We stress that the particular values of r_R and Z_R used to calculate the curves plotted in Fig. 7, *A* and *B*, are reasonable, but not uniquely determined by the data. Essentially identical curves may be calculated using many different pairs of values (r_R, Z_R) as shown in Fig. 9. At the present level of resolution our data are consistent with the notion that both K-Cl and Na/H

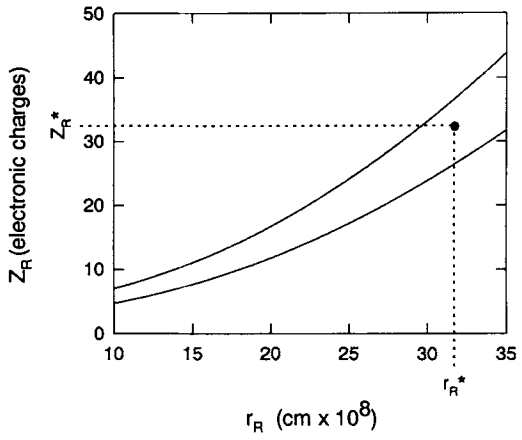

FIGURE 9. Contour map of model parameters consistent with experimentally measured dependence of set point %cw on external salt concentration. *Any* point in the region between the two curves, such as the arbitrarily selected point indicated in the figure, is defined by two coordinate values (r_R^*, Z_R^*) corresponding to values of the parameters r_R and Z_R that generate, via the model described in the text, a functional dependence of set point %cw upon external salt concentration that is consistent with the data reported here to within experimental uncertainty.

transporters are controlled by a single regulatory protein. However, the results are also consistent with separate regulators for each transporter. Further development and testing of the model will require identification and characterization of the putative regulatory protein(s) to provide independent estimates of the values of Z_R and/or r_R.

We believe that at its current level of development, the primary value of the model is qualitative rather than quantitative. Given independent information regarding the interaction between a particular solute and proteins in general, the model permits one to predict the effect of introduction of that solute into a cell upon the set point of a volume-sensitive ion transporter, independent of details of the mechanism of regulation of the particular transporter. The model provides us with a simple rule of thumb: to the extent that introduction of a perturbant results in the stabilization of intracellular protein, arising from associations or other attractive interactions between perturbant and protein, set points will be shifted to higher intracellular protein

concentration (lower %cw). Conversely, to the extent that introduction of a perturbant results in the destabilization of intracellular protein, arising from significant volume exclusion or other repulsive interactions, set points will be shifted to lower intracellular protein concentration (higher %cw). In the present study, increasing the concentration of small intracellular electrolytes, i.e., increasing intracellular ionic strength, shifted set points for the two transporters to lower %cw by lowering γ_R^{SM}.

In another pertinent example, we have previously demonstrated shifting of the set points for volume-activated Na/H exchange and K-Cl cotransport to lower cell water content in response to the introduction of intracellular urea or thiocyanate (Parker, 1993*a*; Parker and Colclasure, 1992). According to the model presented here, these observations suggest that the addition of urea or thiocyanate lowers γ_R^{SM}. This is consistent with reported observations of attractive (stabilizing) interactions between both urea and thiocyanate and a number of proteins (Prakash, Loucheux, Scheufele, Gorbunoff, and Timasheff, 1981; Arakawa and Timasheff, 1982).

APPENDIX I

Calculation of the Intracellular Concentration of Hemoglobin as a Function of %cw

Let %cw denote the percentage of cell mass (weight) that is water. Then $f_w = 0.01\%cw$ is the weight fraction of water. We assume, as stated in the text, that the mass of all other cell constituents, including membranes, is small ($\leq 2\%$) relative to the masses of cell water and hemoglobin. Then 1 g of cells contains f_w g water and $(1\text{-}f_w)$ g hemoglobin, and the volume of this quantity of cells is V (ml) $= f_w\bar{v}_w + (1\text{-}f_w)\bar{v}_{Hb}$, where $\bar{v}_w$ and $\bar{v}_{Hb}$, the partial specific volumes of water and hemoglobin, are taken to be equal to 1.0 and 0.73 ml/g respectively (Attri and Minton, 1983). If the molar mass of hemoglobin is taken as 68,000 g then it follows that the molar concentration of hemoglobin in cells is

$$c_{Hb} = \frac{1}{68}\frac{1 - f_W}{0.73 + 0.27 f_W}. \tag{A1.1}$$

The reciprocal relation is

$$f_W = \frac{1 - 49.64 c_{Hb}}{1 + 18.36 c_{Hb}} \tag{A1.2}$$

APPENDIX II

Additional Small Intracellular Electrolytes

To calculate the charge on hemoglobin, it was necessary to take into account the major small electrolytes in addition to Na, K, and Cl, which had been measured. The additional electrolytes known to be significant in dog red blood cells, concentrations taken from the literature, are 2,3-diphosphoglycerate (2,3-DPG), ATP, and Mg. None of the glycolytic intermediates or other nucleotides are significant; orthophosphate is removed by washing. Total Mg concentration in dog red blood cells is 4.49 mmol/liter cell water (Parker et al., 1989); total ATP is 0.82 mmol/liter cell water (Parker and Snow, 1972); total 2,3-DPG is 9.38 mmol/liter cell water (Bunn, 1971).

Essentially the same concentrations of 2,3-DPG and ATP were recently measured in dog red cells (T.J. McManus, unpublished results). It was assumed that all ATP is Mg-ATP because [Mg] > [ATP] and the K_D for Mg-ATP is small, 0.05 mM. Mg-DPG, DPG, and Mg concentrations were calculated from the K_D for Mg-DPG, 1.5 mM. The net charge of free 2,3-DPG in red cells is −3.5 (Bunn, Forget, and Ranney, 1977). Full oxygenation of the red cell suspensions and the resultant negligible binding of 2,3-DPG to hemoglobin were assumed (Benesch and Benesch, 1969). The calculated free [Mg], 0.87 mmol/liter cell water, or 0.64 mmol/liter cells, is close to the measured value for free [Mg] in dog red cells, 0.5 mmol/liter cells (Parker et al., 1989). Creatine and creatine phosphate (creatine-P) are present in dog red cells (Colclasure and Parker, 1993), but their concentrations have not been measured. They are assumed to be severalfold higher than the creatine + creatine-P concentration in human red cells, 0.4 mmol/liter cells (Griffiths and Fitzpatrick, 1967); creatine kinase is much higher in dog than in human red cells (Colclasure and Parker, 1993). The K_D for Mg-creatine phosphate is 30 mM, so its concentration is insignificant. The concentrations of these various small electrolytes are summarized in Table I. These standard values are normalized to 67 %cw, and converted to units of mmol/liter cell

TABLE I

Concentrations of Small Electrolytes in Dog Red Cells Normalized to 67% Cell Water

Solute	Concentration *mmol/liter cell water*	Net charge/molecule
2,3-DPG	6.58	−3.5
Mg-DPG	2.80	−1.5
ATP	0	—
Mg-ATP	0.82	−1.5
Creatine	1.36	−1.0
Creatine-P	1.36	−2.0
Mg	0.87	+2.0

water. Because the red cell membrane is impermeant to these electrolytes, their concentrations are functions of %cw only and do not vary with external salt concentration at fixed %cw. The concentration of a given electrolyte X (per unit volume of cell water) at an arbitary value of f_w, relative to the standard value at f_w^o = 0.67, is given by

$$\frac{c_X}{c_X^o} = \frac{f_W^o}{1 - f_W^o}\frac{1 - f_W}{f_W} \simeq 2\frac{1 - f_W}{f_W} \qquad \text{(A2.1)}$$

It was assumed that charge of the small electrolytes and the K_D's for Mg-ATP and Mg-DPG did not change with changes in %cw.

APPENDIX III

Calculation of Net Charge on Intracellular Hemoglobin

The condition of electroneutrality may be expressed as

$$V_{tot}c_{Hb}Z_{Hb} + V_W\left(\sum_{cation} c_{cation}Z_{cation} + \sum_{anion} c_{anion}Z_{anion}\right) = 0 \qquad \text{(A3.1)}$$

where V_{tot} denotes the total cellular volume, V_W denotes the volume of cell water,[1] and Z_i denotes the electronic charge per molecule of the ith species. The species considered were Na, K, Cl, and those listed in Table I (Appendix II). It follows from Eq. A3.1 and Appendix I that

$$Z_{Hb} = -\frac{V_W}{V_{tot}} \frac{\sum_{cation} c_{cation} Z_{cation} + \sum_{anion} c_{anion} Z_{anion}}{c_{Hb}} \tag{A3.2}$$

where

$$\frac{V_W}{V_{tot}} = \frac{f_W}{f_W + 0.73(1 - f_W)}.$$

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[1] The distinction between the two volumes is made because hemoglobin concentration is calculated with respect to total cell volume and small molecule concentrations are calculated with respect to cell water volume.

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