Proton (or Hydroxide) Fluxes and the Biphasic Osmotic Response of Human Red Blood Cells

JOHN D. BISOGNANO, JAMES A. DIX, PROMOD R. PRATAP, TERRI S. NOVAK, and JEFFREY C. FREEDMAN

From the Department of Chemistry, State University of New York, Binghamton, New York 13902-6000; and the Department of Physiology, State University of New York Health Science Center, Syracuse, New York 13210

ABSTRACT Upon exposure of human red blood cells to hypertonic sucrose, the fluorescence of the potentiometric indicator 3,3′-dipropylthiadicarbocyanine iodide, denoted diS-$C_3(5)$, displays a biphasic time course indicating the rapid development of an inside-positive transmembrane voltage, followed by a slow DIDS (4,4′-diisothiocyano-2,2′-disulfonic acid stilbene)-sensitive decline of the voltage. In addition to monitoring membrane potential, proton (or hydroxide) fluxes were measured by a pH stat method, cell volume was monitored by light scattering, and cell electrolytes were measured directly when red cells were shrunken either with hypertonic NaCl or sucrose. Shrinkage by sucrose induced an initial proton efflux (or OH^- influx) of 5.5 μeq/g Hb·min and a Cl shift of 21–31 μeq/g Hb in 15 min. Upon shrinkage with hypertonic NaCl, the cells are initially close to Donnan equilibrium and exhibit no detectable shift of Cl or protons. Experiments with the carbonic anhydrase inhibitor ethoxzolamide demonstrate that for red cell suspensions exposed to air and shrunken with sucrose, proton fluxes mediated by the Jacobs-Stewart cycle contribute to dissipation of the increased outward Cl concentration gradient. With maximally inhibitory concentrations of ethoxzolamide, a residual proton efflux of 2 μeq/g Hb·min is insensitive to manipulation of the membrane potential with valinomycin, but is completely inhibited by DIDS. The ethoxzolamide-insensitive apparent proton efflux may be driven against the electrochemical gradient, and is thus consistent with HCl cotransport (or Cl/OH exchange). The data are consistent with predictions of equations describing nonideal osmotic and ionic equilibria of human red blood cells. Thus osmotic equilibration after shrinkage of human red blood cells by hypertonic sucrose occurs in two time-resolved steps: rapid equilibration of water followed by slower equilibration of chloride and protons (or hydroxide). Under our experimental conditions, about two-thirds of the osmotically induced apparent proton efflux is mediated by the

Address reprint requests to Dr. Jeffrey C. Freedman, Department of Physiology, SUNY Health Science Center, 750 East Adams Street, Syracuse, NY 13210.

Dr. Bisognano's present address is Department of Internal Medicine, University of Michigan Medical Center, Ann Arbor, MI 48109.

J. GEN. PHYSIOL. © The Rockefeller University Press · 0022-1295/93/07/0099/25 $2.00
Volume 102 July 1993 99–123

Jacobs-Stewart cycle, with the remainder being consistent with mediation via DIDS-sensitive HCl cotransport (or Cl/OH exchange).

INTRODUCTION

During the course of their normal circulation, human red blood cells are subjected to hypertonicity in the renal medulla. Quantitative understanding of the kinetics of the red cell osmotic response is important in cases of sickle cell anemia because during the transit time of red cells in the renal medulla hypertonicity concentrates intracellular hemoglobin, thus promoting polymerization of sickle hemoglobin and cell sickling, which in turn causes vaso-occlusion and renal dysfunction (Perillie and Epstein, 1963). The response of human red cells to osmotic stress has long been known to be thermodynamically nonideal (Ponder, 1948; Dick and Lowenstein, 1958; Savitz, Sidel, and Solomon, 1964; Freedman and Hoffman, 1979*a*; Solomon, Dix, and Toon, 1986). In a previous study, the classical equations for red cell ionic and osmotic equilibria (Van Slyke, Wu, and McLean, 1923; Jacobs and Stewart, 1947) were rewritten to include the osmotic coefficient of hemoglobin, ϕ_{Hb}, and also the nonideal activity and osmotic coefficients of other solutes; the resultant nonideal thermodynamic model predicted equilibrium water contents of nystatin-treated red cells with an average deviation of 2.4% from the measured values (Freedman and Hoffman, 1979*a*). In osmotic experiments with red cells having normal cation permeability, a significant systematic discrepancy of $<4\%$ between the measured and predicted cell volumes prompted the suggestion (Solomon et al., 1986) that the dependence of ϕ_{Hb} on ionic strength may account for part of the small remaining disparity between theory and experiment.

When red cells are shrunken with an impermeant nonelectrolyte such as sucrose, rapid net efflux of water concentrates intracellular Cl, thus creating a transient outwardly directed Cl concentration gradient. In the absence of significant fluxes of K, Na, or other cations, reequilibration of Cl occurs primarily by exchange with bicarbonate via the Jacobs-Stewart cycle (Jacobs and Stewart, 1942). Some years ago, we observed that the electrical response of red cells to hypertonic sucrose follows a biphasic time course and includes rapid development of an inside-positive membrane potential, which is then slowly and partially dissipated (Freedman and Hoffman, 1977). Light-scattering traces intended to monitor red cell volume changes in response to hypertonic sucrose are also biphasic (Dix, J. A., and A. Solomon, unpublished observations). The experiments described in this paper extend and explain these observations by demonstrating that the resultant net efflux of Cl is accompanied by a chemically coupled efflux of protons (or, indistinguishably, by an influx of hydroxide) and by nonideal water movements. Further experiments with the carbonic anhydrase inhibitor ethoxzolamide indicate that the osmotically induced proton fluxes are mediated by two pharmacologically distinct pathways: (*a*) the Jacobs-Stewart cycle and (*b*) a second pathway which is insensitive to ethoxzolamide and to manipulation of the transmembrane voltage with valinomycin, but which is completely inhibited by the anion transport inhibitor 4,4′-diisothiocyano-2,2′-disulfonic acid stilbene (DIDS). The second pathway is capable of mediating apparent proton fluxes against the electrochemical gradient and is thus consistent with HCl cotransport (or Cl/OH exchange). Such proton (or hydroxide) fluxes have not

previously been implicated or appropriately modeled in the osmotic responses of human red blood cells. Quantitative analysis of the experiments in this paper thus enhances understanding of the complex relationships describing membrane potential, proton and Cl fluxes, and nonideal water movements during the common experimental procedure of osmotically stressing human red blood cells with hypertonic NaCl or sucrose.

Some of these results were briefly described previously (Bisognano and Dix, 1984; Bisognano, Dix, and Freedman, 1990).

MATERIALS AND METHODS

Preparation of Cells and Reagents

Blood from healthy human donors was collected into heparinized tubes. After centrifuging for 3–5 min at 12,000 *g* and 4°C, the plasma and buffy coat were aspirated and discarded and the packed cells were then washed three or four times by centrifugation in 3–5 vol of cold isotonic medium as specified in the figure captions. Stock cell suspensions were prepared by diluting the packed cells to 50% hematocrit (HCT) for fluorescence and light scattering measurements and to 72% HCT for pH-stat experiments, and were kept on ice for use on the same day.

In some experiments, cells were treated with 10 μM DIDS (Sigma Chemical Co., St. Louis, MO) by incubation at 0.17% HCT for 30 min at 25°C, followed by two washes in medium without DIDS. Two washes sufficed to remove the quenching of the fluorescence of 3,3′-dipropylthiadicarbocyanine iodide (diS-C_3(5)) by DIDS. A stock solution of 10 mM DIDS in 20 mM NaOH was prepared on the day of the experiment. Other stock solutions were bovine erythrocyte carbonic anhydrase (Sigma Chemical Co.) at 30,000 U/ml in isotonic unbuffered medium, valinomycin (Calbiochem Corp., La Jolla, CA) at 2.5 mM in ethanol, and ethoxzolamide at 1 mM, also in ethanol.

Membrane Potential and Light Scattering

Membrane potentials were monitored by the fluorescence of the cationic dye diS-C_3(5) (Molecular Probes, Inc., Eugene, OR) as described elsewhere (Freedman and Novak, 1989). The excitation wavelength was 616 nm (16-nm bandpass) and the emission wavelength was 676 nm (8-nm bandpass). An RG665 cut-off filter (Schott Optical Glass, Inc., Duryea, PA) was placed in front of the emission monochromator. All samples were thermostatted at 23°C. A small hole drilled in the sample chamber lid permitted injection of reagents into the cuvette.

Experiments were performed at 0.17% HCT, which was previously found to optimize the sensitivity of monitoring changes in the membrane potential of human red blood cells with diS-C_3(5) (Freedman and Hoffman, 1979*b*). The dye was added to the cell suspensions in acrylic cuvettes (Sarstedt, Inc., Newton, NC) from a stock solution (0.2 mg/ml ethanol) to a final concentration of 1 μM. The cuvettes were stirred with a teflon-coated magnetic bar. After adding sucrose, the cell suspension was stirred for 5 s with a plastic paddle to ensure complete mixing. A slow decrease of fluorescence intensity in the cell suspensions, presumably due to binding of dye to the cuvette and stir bar, averaged -0.7 ± 1.1 $\%\Delta F$/min (SD, $n = 30$). In each experiment, the drift was measured during 1 min before the addition of reagents, and these values were used to correct the computed values for the percentage changes in fluorescence that occurred after addition of hypertonic sucrose or NaCl.

The initial percent increase of diS-C_3(5) fluorescence, $\%\Delta F_i$, was measured 1 min after addition of NaCl, or at the maximum amplitude of fluorescence after shrinkage with sucrose

(~1 min). With corrections for drift and for dilution by the added reagents,

$$\%\Delta F_i = 100\cdot[(F_3 - F_2)/F_2 - (F_2 - F_1)/F_2 + 0.034]\cdot 1.04 \quad (3)$$

where F_2 is the fluorescence intensity immediately before addition of sucrose or NaCl, F_1 is the fluorescence intensity 1 min before the addition, and F_3 is the peak fluorescence intensity after the addition. The expression $100\cdot(F_3 - F_2)/F_2$ is the uncorrected percent change in fluorescence. The term $100\cdot(F_2 - F_1)/F_2$ corrects for drift. The factor 0.034 corrects for dilution, as determined in nine experiments by adding a volume of buffer equivalent to that of the sucrose or NaCl. The factor 1.04, also determined from the buffer controls, refers the values of $\%\Delta F_i$ to the new baseline after dilution. The percent decline in fluorescence, $\%\Delta F_s$, during 5 min after cell shrinkage was calculated from

$$\%\Delta F_s = 100\cdot[(F_4 - F_3)/F_2 + 0.025]\cdot 1.04 \quad (4)$$

where F_4 is the fluorescence intensity 6 min after the addition of sucrose. The factor 0.025 corrects for drift during the equivalent 5 min in the buffer controls. The data are presented as means ± SD for n experiments, each of which usually included three repetitions for each condition.

Traces of fluorescence intensity vs. time were transferred to a microcomputer using the program Sigmascan and a digitizing tablet (Jandel Scientific, Corte Madera, CA), and were then exported to the spreadsheet in Sigmaplot (Jandel Scientific). Each trace was normalized to the baseline value attained before addition of sucrose, salt, or buffer.

Cell volume changes were monitored by 90° light scattering, which was measured simultaneously with fluorescence using the T-format of the SLM 8000S fluorimeter. To distribute the light evenly to the photomultiplier tube, a quartz scattering plate (SLM-AMINCO, Urbana, IL) was placed between the sample cuvette and the detector.

Proton (Hydroxide) Fluxes

A pH stat apparatus (model ETS 822; Radiometer, Copenhagen, Denmark) was used to monitor proton (or hydroxide) fluxes in suspensions of red cells either exposed to air or in a controlled gas environment. Since proton efflux is indistinguishable from hydroxide influx (see Appendix), we shall refer to this flux for convenience as apparent proton efflux. A water-jacketed beaker, attached to a constant temperature water circulator set at 25°C, was fitted with a neoprene stopper through which protruded flexible pH and reference electrodes (Microelectrodes Inc., Londonderry, NH), the delivery tip from an automatic burette, and injection and gas-inlet ports. Unbuffered isotonic medium (26.25 ml) was added to the beaker, followed by 75 μl of 72% HCT red cells, and the suspension was allowed to equilibrate for ~10 min. With the proportional band selector set to 0.05 and the rate of addition of titrant set to the maximum of 160, the resultant pH was then maintained within ±0.01–0.05 pH units by the pH stat, while the pH and the volume of NaOH added during this baseline period and thereafter were both recorded simultaneously. Unbuffered isotonic or hypertonic solution, whose pH was adjusted with 20–50 μM Trizma base to within 0.05 pH units of the maintained pH, was then injected into the beaker. During 1–3 min after this addition, the volume of NaOH added by the pH stat was taken as a measure of apparent proton efflux across the red cell membrane. To normalize the fluxes, the Hb content of each cell suspension was also measured, as described below. NaOH was prepared fresh, stored, and standardized as recommended by Skoog and West (1963); the molarity was 10.1 ± 0.1 mM (SD, n = 3) when first prepared, and 10.3 ± 0.1 mM (SD, n = 3) at the conclusion of the series of experiments. The apparent proton effluxes (μeq/g Hb·min) were computed from $V_T\cdot C_T/30\cdot \text{Hb}\cdot t$, where V_T (milliliters) and C_T (microequivalents per milliliter) are the volume and concentration of titrant, Hb is the hemoglobin

concentration (grams per milliliter) of the suspension, t is the time (minutes) during which the flux was determined, and 30 is the final volume (milliliters) of the suspension.

Cell Cl, K, Na, Water Content, and Hemoglobin

Cells were sedimented by centrifuging at 3,000 rpm for 5 min in 5 or 10 ml polypropylene syringe tubes to which 0.4-ml polycarbonate microcentrifuge tubes had been attached (Freedman and Hoffman, 1979*a*). The syringe tube containing most of the supernatant was removed and discarded. The cells were hemolyzed in 5 ml distilled water and the hemolysates were diluted appropriately for analyses of Cl, K, Na, and Hb. K and Na were measured by flame photometry using 15 mM LiCl as internal standard (Freedman and Hoffman, 1979*a*). Chloride was estimated with a Buchler-Cotlove chloridometer (Buchler Instruments, Inc., Fort Lee, NJ). Samples of cells were dried in a vacuum oven for 18–20 h at 105°C to determine total cell water content. Hb concentration was measured with Drabkin's reagent using cyanmethemoglobin standards.

Computer simulations according to the nonideal thermodynamic model for red cell ionic and osmotic equilibria (Freedman and Hoffman, 1979*a*) were performed using our program IONIC-F, now rewritten in Turbo-Pascal for the IBM microcomputer. The results were found to agree precisely with a version written in Basic (Solomon et al., 1986).

RESULTS

Osmotically Induced Changes in Membrane Potential

When human red blood cells in isotonic medium are equilibrated with the fluorescent potentiometric indicator diS-$C_3(5)$, and then shrunken by adding hypertonic sucrose to a final concentration of 300 mM, the dye fluorescence first rises rapidly and then declines slowly over a period of 15 min (Fig. 1 *A*, top trace). In contrast, cells pretreated with 10 μM DIDS and then shrunken with sucrose show a monophasic increase in dye fluorescence to a new steady level (Fig. 1 *A*, middle trace). With the cationic dye diS-$C_3(5)$, relative to dilution and other controls, increases in suspension fluorescence indicate a membrane potential more positive inside, while decreases in fluorescence indicate a more negative potential (Freedman and Hoffman, 1979*b*; Freedman and Novak, 1989). Exposure of the cells to a hypertonic solution of an impermeant nonelectrolyte such as sucrose concentrates intracellular Cl, resulting in the rapid development of a transmembrane voltage more positive inside which then declines as the increased [Cl] is dissipated by the available pathways. Since DIDS inhibits Cl conductance, the membrane potential is expected to become less positive upon cell shrinkage in the presence of the inhibitor than in its absence, and also more stable, in accordance with the fluorescence traces in Fig. 1 *A*.

In contrast to the results with sucrose, cells shrunken by adding hypertonic NaCl or KCl to 150 mM above the isotonic concentration show a rapid decrease in dye fluorescence (Fig. 1 *B*, lower two traces) relative to a control for dilution of the dye obtained by adding an equivalent volume of isotonic buffer (Fig. 1 *B*, upper trace), with little subsequent change in fluorescence. In this case, the increase in extracellular Cl nearly offsets the increase in intracellular Cl, resulting in very little change in membrane potential as indicated by the traces in Fig. 1 *B* as compared with those in Fig. 1 *A*.

Several control experiments tested for possible artifacts that might contribute to

the biphasic time course seen after shrinkage with sucrose (Fig. 1 *A*, top trace). When the cell suspensions were diluted with a volume of buffer equivalent to that of the added sucrose, the fluorescence intensity was stable for at least 30 min after the initial perturbation due to dilution of the dye (Fig. 1, *A–D*). Shrinkage with impermeant Na-gluconate or Na-citrate also produced biphasic time courses (Bisognano, 1984), indicating that the biphasic response was not peculiar to sucrose and could be obtained over a range of extracellular ionic strengths. Without cells, the fluorescence of dye in the medium alone decreased by the expected amount due to dilution when

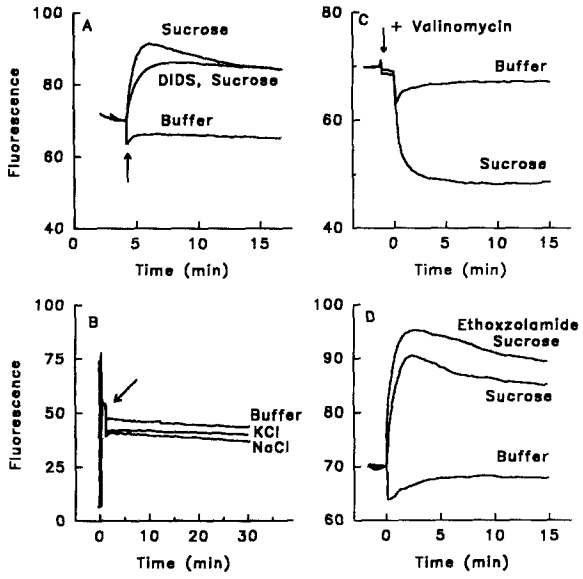

FIGURE 1. Changes in diS-$C_3(5)$ fluorescence upon shrinkage of human red blood cells with (*A*) hypertonic sucrose with or without pretreatment with 10 μM DIDS (see Materials and Methods), (*B*) hypertonic NaCl or KCl, (*C*) hypertonic sucrose after addition of 1 μM valinomycin (at arrow), and (*D*) hypertonic sucrose with and without prior addition of 5 μM ethoxzolamide. For *A*, *B*, and *D*, the cuvettes contained 2.19 ml of medium consisting of 5 mM KCl, 145 mM NaCl, and 5 mM HEPES buffer, adjusted with NaOH to pH 7.4 at 25°C; for *C*, the medium contained 100 mM KCl, 50 mM NaCl, and 5 mM HEPES, pH 7.4. Washed 50% HCT red cells (8.5 μl) were added, followed by addition of 6.8 μl diS-$C_3(5)$ to a final dye concentration of 1 μM. For *A*, *C*, and *D*, the traces begin after equilibration of cells with dye for 5–7 min, after which 0.31 ml of 2.4 M sucrose in the above media was added, bringing the total volume to 2.5 ml with a final sucrose concentration of 300 mM. Addition of sucrose was at the arrow in *A*, and where the fluorescence changes abruptly in *C* and *D*. For *B*, 0.31 ml of 1.2 M KCl or NaCl in the medium was added (at arrow) instead of sucrose, bringing the final salt concentration to 150 mM (300 ideal mosM) above isotonic. The initial spike in *B* shows equilibration of dye with cells. In each panel 0.31 ml of buffer alone was added as a control.

the same volume of medium or hypertonic sucrose was added, indicating that sucrose and the change in viscosity due to addition of sucrose have no detectable effects on the fluorescence of diS-$C_3(5)$ (not shown). Artifacts due to changes in the index of refraction are unimportant since the light-scattering component of the fluorescence signal is negligible. Control traces upon addition of buffer alone were identical with or without pretreatment of the cells with DIDS (not shown).

In addition to the experiment with DIDS (Fig. 1 *A*, middle trace), another control experiment also defined a condition in which dye fluorescence is stable in the

presence of hypertonic sucrose. The slow decline of fluorescence is abolished when cells in isotonic medium at 100 mM $[K]_o$ are exposed to the K ionophore valinomycin before shrinkage with hypertonic sucrose (Fig. 1 *C*, lower trace). Under these conditions the membrane voltage is dominated by the potassium ion diffusion potential, which at the null point of 100 mM $[K]_o$ is close to the −7-mV resting potential of the untreated cells. Consequently, a negligible change in the fluorescence of diS-$C_3(5)$ is observed upon addition of valinomycin (Fig. 1 *C*, arrow). Upon subsequent addition of 300 mM sucrose, the intracellular concentration of K increases, resulting in an increased inside-negative potential with a stable decrease in dye fluorescence. The fluorescence is stable after shrinkage in the presence of valinomycin (Fig. 1 *C*) because the membrane potential and the dye are no longer sensitive to the changing intracellular concentration of Cl. In three such experiments with addition of 300 mM sucrose after valinomycin, the initial percentage change in fluorescence, denoted $\%\Delta F_i$, was -24 ± 2 (SD); the subsequent change in fluorescence during 5 min, denoted $\%\Delta F_s$, was negligible at $+4.9 \pm 5.0$ (SD). This experiment with valinomycin (Fig. 1 *C*), and the result with DIDS (Fig. 1 *A*, middle trace), both indicate that diS-$C_3(5)$ does respond to changes in membrane potential in red cells exposed to hypertonic sucrose, that the resultant fluorescence can be stable in the presence of sucrose, and that artifacts such as bleaching of the dye or quenching of its fluorescence by possible interactions between the dye and the extracellular reagents are not the cause of the declining part of the biphasic response (Fig. 1 *A*, top trace). Moreover, depending on whether the dominant membrane conductance is due to Cl (Fig. 1 *A*) or to K (Fig. 1 *C*), osmotic shrinkage by sucrose results in a transmembrane voltage that is either more positive (Fig. 1 *A*) or more negative (Fig. 1 *C*).

In human red blood cells, proton fluxes can be mediated by the Jacobs-Stewart cycle, a pathway involving Cl/HCO_3 exchange in series with carbonic acid equilibria catalyzed by intracellular carbonic anhydrase and with CO_2 equilibration (Jacobs and Stewart, 1942; see Appendix and Fig. 7 *A*). From the stopped-flow experiments of Critz and Crandall (1980) on pH equilibration, it was unclear whether the Jacobs-Stewart cycle functions appreciably in suspensions of washed red cells exposed to air, where the bicarbonate concentrations are estimated to be <100 μM, some two orders of magnitude less than the K_m of 10 mM for bicarbonate transport mediated by the red cell anion exchanger capnophorin (Wieth, 1979). To check whether proton fluxes mediated by the Jacobs-Stewart cycle contribute to the dissipation of the osmotically induced positive membrane potential, the experiments with sucrose were repeated in the presence and absence of 5 μM ethoxzolamide, a maximally inhibiting concentration of this potent and permeant inhibitor of red cell carbonic anhydrase (Wieth, 1979). The results in Fig. 1 *D* indicate that inhibition of carbonic anhydrase with ethoxzolamide is associated with an increased fluorescence of diS-$C_3(5)$ upon shrinkage with 300 mM sucrose, corresponding to the development of a more positive membrane potential than in the absence of the inhibitor. In another experiment with [sucrose] ranging from 150 to 600 mM, the osmotically induced fluorescence changes with ethoxzolamide averaged 24% greater than in the absence of inhibitor (not shown). Ethoxzolamide did not affect the potentials developed with sucrose in cells treated with valinomycin (not shown). The more

positive potential attained with ethoxzolamide is consistent with participation of the Jacobs-Stewart cycle in contributing to the dissipation of the osmotically induced increase in the outward Cl gradient. In contrast to the stable fluorescence level seen with cells pretreated with DIDS (Fig. 1 *A*), dye fluorescence in the presence of maximally inhibitory ethoxzolamide still shows a biphasic time course (Fig. 1 *D*), suggesting that a pathway other than the Jacobs-Stewart cycle may also contribute to dissipation of the increased outward Cl concentration gradient.

With cells exposed to sucrose concentrations ranging from 0 to 600 mM, the changes in the fluorescence of diS-$C_3(5)$ were quantified by measuring the maximal fluorescence intensity that was reached ~1 min after osmotic shrinkage (Fig. 2 *A*),

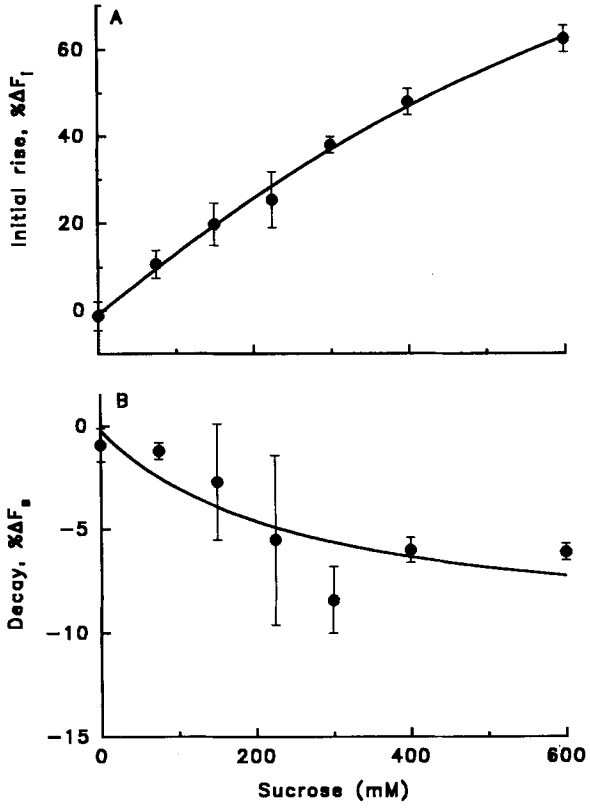

FIGURE 2. Effect of varied [sucrose] on initial increase in diS-$C_3(5)$ fluorescence (*A*) and on the subsequent decay of fluorescence during 5 min (*B*). Experiments were performed as described in Fig. 1 *A*, *top trace*, except with 0–600 mM [sucrose]. The initial percent increase, $\%\Delta F_i$, and subsequent percent decay, $\%\Delta F_s$, were calculated as described in Materials and Methods. Points with error bars represent means ± SD for three experiments, each of which included three trials at each [sucrose].

and also by measuring the subsequent decline of fluorescence between 1 and 6 min after shrinkage (Fig. 2 *B*). The initial percentage increase in fluorescence, or $\%\Delta F_i$, averaged +32 ± 8 $\%\Delta F_i$ (SD, n = 12) with 300 mM sucrose and −19 ± 4 $\%\Delta F_i$ (SD, n = 6) with 150 mM NaCl. The rate of decline of fluorescence during 5 min after shrinkage with 300 mM sucrose was −7 ± 2 $\%\Delta F_s$ (SD, n = 12), substantially different from the negligible values of +2 ± 2 $\%\Delta F_s$ (SD, n = 6) obtained after shrinkage with 150 mM NaCl, and +5 ± 5 $\%\Delta F_s$ (SD, n = 3) for shrinkage with sucrose after pretreatment with valinomycin. Both the initial rise in fluorescence (Fig. 2 *A*) and the

subsequent rate of decline (Fig. 2 *B*) increased as the osmotically induced outward Cl concentration gradient was increased with 0–600 mM sucrose.

Light Scattering

To compare the time courses of changes in cell volume after shrinkage with hypertonic sucrose and NaCl, 90° light scattering was monitored in some experiments simultaneously with the fluorescence of diS-$C_3(5)$. Addition of hypertonic sucrose to a suspension of red cells changes the index of refraction of the medium by an extent which differs from that caused by addition of NaCl; therefore, the initial changes in the intensity of scattered light could not be compared directly. However, all treatments that decreased cell volume increased the intensity of scattered light. Cells shrunken with sucrose showed a continued increase in scattered light intensity between 1 and 6 min after adding sucrose (Fig. 3, middle trace), indicating that the cells continue to shrink during the time that the fluorescence of diS-$C_3(5)$ is

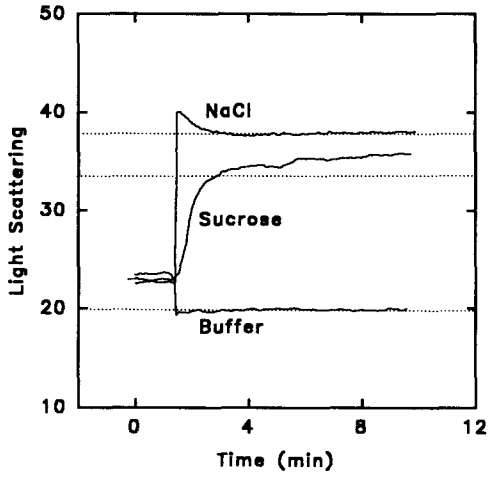

FIGURE 3. Changes in light scattering upon shrinkage of human red blood cells with hypertonic sucrose (300 mM) or NaCl (150 mM) and dilution control. Experiments were performed as described in Fig. 1, *A* (*top trace*) and *B*. Light scattering at 90° was monitored simultaneously with diS-$C_3(5)$ fluorescence (see Materials and Methods). Horizontal dotted lines are included to enable visualization of the upward drift with sucrose.

declining. However, cells shrunken with NaCl do not continue to shrink, but appear to swell slightly and then maintain a stable volume after the initial shrinkage (Fig. 3, top trace). Pretreatment with valinomycin resulted in an increased rate of change of scattered light intensity between 1 and 5 min after shrinkage (Bisognano, 1987), presumably due to increased net efflux of KCl from the shrinking cells. Due to the high viscosity of the 2.4 M sucrose stock solution and the difficulty of achieving rapid mixing in the cuvette, no significance should be attributed to the slow time course of the initial rise of the light-scattering signal upon shrinkage with sucrose (Fig. 3, middle trace). Although changes in index of refraction precluded straightforward quantitative analysis of light scattering, a positive correlation between the rate of increase of light-scattering intensity during 5 min after the initial shrinkage and [sucrose] was also observed (Bisognano, 1987).

The light-scattering changes (Fig. 3), the dependence of the changes in fluorescence on the extent of shrinkage as determined by the external [sucrose] (Fig. 2), the

stable responses obtained upon shrinkage after pretreatment with DIDS (Fig. 1 *A*, middle trace) or with valinomycin (Fig. 1 *C*, lower trace), and the results with ethoxzolamide (Fig. 1 *D*) all indicate that the biphasic response of diS-$C_3(5)$ (Fig. 1 *A*, top trace) is not some optical artifact but is indeed related to changes in membrane potential associated with the Cl concentration gradient.

Cell Electrolyte and Water Contents

Intracellular Cl, Na, K, and water contents were measured initially and 15 min after shrinkage with sucrose or NaCl (Fig. 4). Control cells without additives, or with addition of buffer alone, showed no significant change in [Cl], [Na], [K], or water content for at least 15 min. Cells shrunken with either NaCl or sucrose showed no significant change in Na or K contents during 15 min. In two experiments, cells shrunken with 300 mM sucrose showed significant ($P < 0.001$) net effluxes of Cl of −21 and −31 μeq/g Hb·15 min, while cells shrunken with NaCl showed a significant

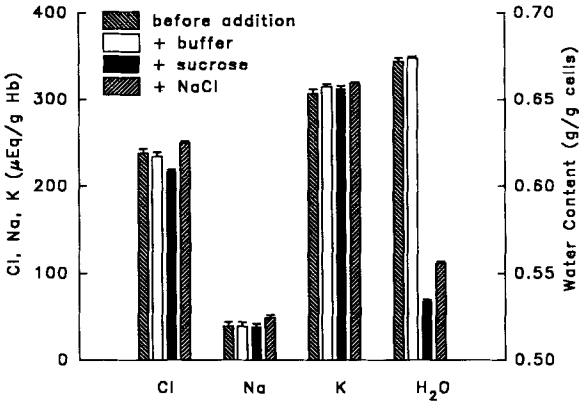

FIGURE 4. Cl, Na, K, and water contents of cells before and after shrinkage with hypertonic sucrose or NaCl. Cell electrolyte and water contents were measured after initial equilibration of washed cells for 15 min at 3% HCT and at 25°C in medium containing 5 mM KCl, 145 mM NaCl, and 5 mM HEPES, pH 7.4 at 25°C. Sucrose (300 mM), NaCl (150 mM), or an equivalent volume of buffer was then added, and the cells were sampled again 15 min later. Each bar represents the average of six determinations of electrolytes or triplicate measurements of water content as described in Materials and Methods; error bars represent ± 1 SD.

increase ($P < 0.001$) in Cl relative to the buffer controls. As expected, more water was lost from cells shrunken with sucrose than with NaCl because the osmotic strength of the medium with sucrose was greater than with NaCl (see Discussion and Table III).

Apparent Proton Fluxes

The results described so far indicate a net efflux of Cl from cells exposed to hyperosmotic sucrose, but not from cells exposed to hyperosmotic NaCl. To maintain bulk electroneutrality, the net efflux of Cl must be accompanied either by an equivalent efflux of cations or influx of anions. To test for apparent proton efflux, pH stat experiments were performed by adding freshly prepared and standardized NaOH to an unbuffered suspension of cells so the initial pH of the cell suspension would be maintained during cell shrinkage. The apparent proton effluxes were measured between 1 and 2 min after adding sucrose. In the absence of ethoxzol-

amide, cells exposed to air and shrunken with 300 mM sucrose showed an apparent proton efflux of 5.5 ± 0.6 μeq/g Hb·min (Fig. 5 *A*, and Table I, row 1). This apparent proton efflux was unaffected by 1 μM diS-C_3(5) (not shown). Cells shrunken with NaCl or diluted with buffer showed no detectable proton efflux, although a small baseline efflux of 0.3 μeq/g Hb·min was detected before the addition of sucrose (Table I, row 6).

To ascertain the involvement of the Jacobs-Stewart cycle in mediating these apparent proton effluxes, [ethoxzolamide] was varied from 0 to 7.5 μM (Fig. 5 *A*). With a maximally inhibitory [ethoxzolamide] of 5 μM, an ethoxzolamide-insensitive apparent proton efflux of 2.0 ± 0.5 μeq/g Hb·min was still present (Fig. 5 and Table I, row 2). In other experiments the apparent proton fluxes were measured after three

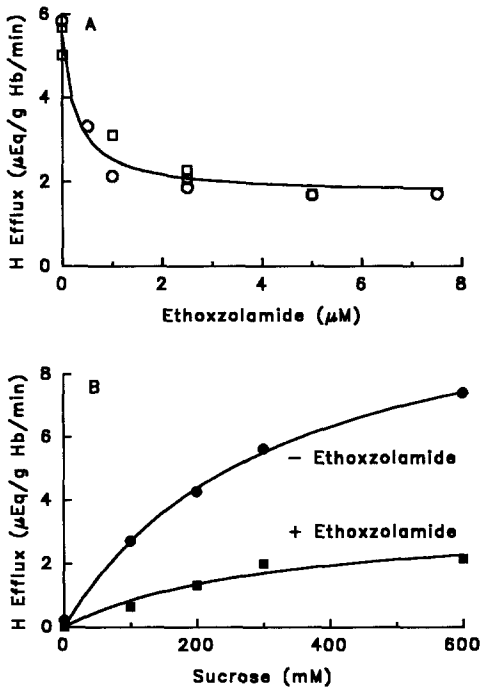

FIGURE 5. Apparent proton effluxes induced by shrinkage with 300 mM sucrose at varied [ethoxzolamide] (*A*) and at 0–600 mM [sucrose] with or without 5 μM ethoxzolamide (*B*). Fluxes were determined as described in Materials and Methods and the legend to Table I, but without addition of valinomycin or pretreatment with DIDS. Open circles and squares (*A*) represent two experiments, while filled symbols (*B*) represent a third experiment. In *B*, the fluxes are corrected by subtracting the baseline flux obtained before addition of sucrose.

to six cycles of evacuation and regassing the sample chamber and all solutions with ultrapure gas containing 80% N_2, 20% O_2, and <3 ppm CO_2 (MG Industries, North Branch, NJ), but an ethoxzolamide-sensitive component persisted, indicating incomplete removal of CO_2. For cells exposed to air, the dependence of these osmotically induced apparent proton effluxes on [sucrose] in the presence and absence of 5 μM ethoxzolamide is shown in Fig. 5 *B*, which again illustrates that proton efflux is composed of ethoxzolamide-sensitive and -insensitive components.

In the absence of ethoxzolamide, addition of 900 U/ml carbonic anhydrase to the extracellular medium doubled the osmotically induced apparent proton efflux, as did addition of 0.3 mM $NaHCO_3$, which also raised the pH from 7.4 to 7.7. When the initial pH was varied over this same range by adding NaOH, the apparent proton

efflux increased by only 14%. These experiments confirm definitively the participation of the Jacobs-Stewart cycle in suspensions of washed red cells exposed to air (cf. Critz and Crandall, 1980).

For cells pretreated with 10 μM DIDS, the apparent proton efflux was completely inhibited to the baseline value either in the presence of ethoxzolamide (Table I, row 4) or in its absence (not shown). Pretreatment with valinomycin at 100 mM K_o had no significant effect ($P > 0.05$) on the apparent proton efflux (Table I; cf. rows 3 and 2). Pretreatment with DIDS completely inhibited the apparent proton efflux to the baseline value for cells that were also treated with valinomycin (Table I, row 5). Thus the ethoxzolamide-insensitive, DIDS-sensitive component of the osmotically induced apparent proton efflux appears to be unaffected by manipulation of the transmembrane voltage with valinomycin. Such insensitivity to voltage is consistent with electrically silent HCl cotransport or Cl/OH exchange.

TABLE I

Osmotically Induced Apparent Proton Efflux Effects of Ethoxzolamide, Valinomycin, and DIDS

Addition	Apparent proton efflux
	μeq/g Hb · min
Sucrose	5.5 ± 0.6
Sucrose, 5 μM ethoxzolamide	2.0 ± 0.5
Sucrose, 5 μM ethoxzolamide, 1 μM valinomycin at 100 mM K_0	1.7 ± 0.6
Sucrose, 5 μM ethoxzolamide, pretreated with 10 μM DIDS	0.4 ± 0.1
Sucrose, 5 μM ethoxzolamide, 1 μM valinomycin at 100 mM K_0, pretreated with 10 μM DIDS	0.2 ± 0.1
Baseline, before addition of sucrose	0.3 ± 0.1

Apparent proton effluxes were measured from the rate of addition of NaOH needed to maintain constant pH (see Materials and Methods) with an additional 30 min allowed for equilibration of pH after addition of ethoxzolamide, but before addition of valinomycin. In each experiment the last addition was 300 mM sucrose. The baseline flux refers to the average apparent proton efflux measured in all of the above conditions before the addition of sucrose. All other values represent means ± 1 SD for three experiments.

Despite the statistical insignificance of the effect of valinomycin on the ethoxzolamide-insensitive apparent proton efflux (Table I, rows 2 and 3), such experiments can only establish an upper limit on the magnitude of any possible proton conductance, and cannot rigorously eliminate the possibility of the existence of a small proton conductance. Another approach to distinguish between electrically coupled conductive fluxes and chemically coupled mediated fluxes is to set the chemical and electrical gradients and then test whether one of the ionic species may be driven against its electrochemical gradient. For this purpose, we note that the isoelectric pH, denoted by pI, is defined as the pH where the net charge on intracellular constituents, primarily Hb and to a lesser extent organic phosphates, is zero. For human red blood cells at a pI of 6.8 at 25°C, the Gibbs-Donnan equilibrium potential is zero and

the ratios of intracellular to extracellular [Cl^-], [HCO_3^-], and [H^+] are all unity (see Freedman and Hoffman, 1979*a*, and references therein). The initial proton concentration gradient is inwardly directed below pI and outwardly directed above pI. Consequently, by setting the extracellular pH to 6.7, just below pI, and allowing sufficient time for pH equilibration, we are assured that any osmotically induced efflux of protons (or influx of hydroxide) will be against the chemical concentration gradient. Control experiments with diS-C_3(5) showed that at pH 6.7, pretreatment of cells with valinomycin at 100 mM $[K]_o$ followed by shrinkage with 300 mM sucrose hyperpolarized the cells (not shown), similar to the results obtained at pH 7.4 (Fig. 1 *C*). Consequently, in a red cell suspension set to pH 6.7 and pretreated with valinomycin, any osmotically induced efflux of protons (or influx of hydroxide) would have to be against the electrochemical potential gradient.

The apparent proton effluxes induced by shrinkage with 300 mM sucrose were elevated at pH 6.7, as compared with pH 7.3 (Table II, rows 1 and 3). With 5 μM

TABLE II

Osmotically Induced Apparent Proton Efflux against the Electrochemical Gradient

Addition	Apparent proton efflux
pH 7.3	*μeq/g Hb · min*
Sucrose	6.2
Sucrose, 5 μM ethoxzolamide	2.3
pH 6.7	
Sucrose	8.6
Sucrose, 1 μM valinomycin at 100 mM K_0	7.5
Sucrose, 5 μM ethoxzolamide	5.4
Sucrose, 5 μM ethoxzolamide, 1 μM valinomycin at 100 mM K_0	4.5

Apparent proton effluxes were measured as described in Materials and Methods and in the legend to Table I, except that where indicated the initial extracellular pH of the cell suspension was adjusted from 7.4 to 6.7 with incremental additions (10 μl each) of 0.01 N HCl over ~30 min. The values given represent averages from two experiments.

ethoxzolamide the apparent proton efflux is only partially inhibited at pH 6.7, as it is at 7.3 (Table II, rows 2 and 5). Controls showed that 5 μM ethoxzolamide was still maximally inhibitory at pH 6.7, as it is at 7.3 (not shown). In the presence of 1 μM valinomycin at pH 6.7 the apparent proton efflux in the presence or absence of ethoxzolamide is comparable to that seen in the absence of the K ionophore (Table II). Thus the ethoxzolamide-insensitive apparent proton efflux persists even under conditions in which protons are apparently moving against both the concentration and electrical gradients (Table II, row 6).

DISCUSSION

The major new findings in this paper are that the readjustment of water, electrolytes, and the transmembrane voltage of human red blood cells after exposure to hypertonic sucrose follows a biphasic time course, that dissipation of the increased

outward Cl concentration gradient involves fluxes of Cl accompanied by protons (or hydroxide), and that the osmotically induced apparent proton effluxes are mediated both by the Jacobs-Stewart cycle and by a pharmacologically distinct pathway that is insensitive to ethoxzolamide and valinomycin, yet inhibitable with DIDS. The persistence of the osmotically induced ethoxzolamide-insensitive apparent proton efflux under conditions where the protons (or hydroxides) are moving against their respective electrochemical gradients is consistent either with HCl cotransport or Cl/OH exchange, and is inconsistent with electrical coupling of conductive fluxes. The time courses of the readjustments of membrane potential (Fig. 1) and cell volume (Fig. 3) are biphasic after osmotic shrinkage because water equilibrates in tenths of seconds (Sidel and Solomon, 1957), while anions equilibrate on a time scale of minutes (Hunter, 1977; Knauf, Fuhrmann, Rothstein, and Rothstein, 1977).

Predictions of the Nonideal Thermodynamic Model

To aid in understanding the results quantitatively, the osmotic properties of human red blood cells were predicted by a nonideal thermodynamic model, previously developed to describe ionic and osmotic equilibria of human red blood cells (Freedman and Hoffman, 1979*a*). The model was utilized for hypertonic shrinkage by sucrose and NaCl. For each substance, the results were calculated for two time scales: one in which only water is in osmotic equilibrium across the membrane, and one in which both water and anions have reached equilibrium. The first column in Table III lists the cell parameters that are either known or calculated[1] from given values for the composition of the extracellular medium and certain cellular constants, as described in the legend. The second column gives the initial values of these parameters in the experimental medium before osmotic perturbation. The third and fourth columns give predicted values for shrinkage by hypertonic NaCl; the fifth and sixth columns give predicted values for shrinkage by hypertonic sucrose. The values in the third and fifth columns were predicted when the model was constrained to allow water movement only, while the values in the fourth and sixth columns were predicted when the model permitted both water and anions to equilibrate.

The model predicts that if red cells are initially suspended in 284 mosM (150 mM) NaCl with 5 mM HEPES, pH 7.4 at 25°C, and are then exposed to hyperosmotic NaCl (555 mosM, 300 mM, pH 7.4), the Donnan ratio for Cl initially decreases from 0.761 to 0.683 as water reequilibrates, but then changes by only 2% to 0.698 as anions reequilibrate (Table III, row 14). In contrast, upon exposure to hyperosmotic sucrose (616 mosM, 300 mM, pH 7.4), the predicted Donnan ratio for Cl increases initially from 0.761 to 1.48 as water equilibrates, and then decays back to 1.27 as anions reequilibrate (Table III, row 14). Thus for shrinkage by sucrose, immediately after equilibration of water but before equilibration of anions, the model predicts an

[1] Due to a typographical error, the equation for the Donnan ratio, r_a, (Eq. 6a of Freedman and Hoffman, 1979*a*) inadvertently was missing an osmotic coefficient Φ_o preceding the first R_a, but the calculations in that paper were done correctly with this term included. The correct equation is as follows:

$$r_a = \frac{\Phi_o R_a E + [Q(D/\gamma_{j,k})^2 + (\Phi_o R_a E)^2 - Q(\varphi_s E/\gamma_{j,k})^2]^{1/2}}{D + \varphi_s E}$$

TABLE III

Predictions of the Nonideal Thermodynamic Model for Osmotic Shrinkage of Human Red Blood Cells

Cell Parameters	Initial	NaCl shrink		Sucrose shrink	
		H_2O	H_2O + Anions	H_2O	H_2O + Anions
Water content					
(g/g isotonic water)	0.967	0.539	0.543	0.495	0.472
(g/g cells)	0.658	0.518	0.520	0.497	0.485
(g/g cell solids)	1.928	1.074	1.083	0.988	0.942
Electrolytes					
Na + K (μeq/g Hb)	321	321	321	321	321
$[Na]_c$ + $[K]_c$ (mmolal)	156	280	278	305	320
Cl (μeq/g Hb)	233	236	242	236	191
$[Cl]_c$ (mmolal)	114	205	209	223	191
[P] (mmolal)	12.5	22.5	22.3	24.4	25.6
[Hb] (mmolal)	7.6	13.5	13.4	14.7	15.5
C_c (mmolal)	290	521	523	567	551
Osmotic balance					
ϕ_{Hb}	2.96	6.61	6.52	7.55	8.16
$\Phi_c C_c$ (mosmolal)	284	555	555	616	616
$\Phi_c C_c / \Phi_o C_o$	1.00	1.00	1.00	1.00	1.00
Donnan ratio	0.761	0.683	0.698	1.48	1.27
Cl potential, E_{Cl} (mV)	−7.0	−9.8	−9.2	+10.1	+6.1
Intracellular pH	7.25	7.25	7.21	7.25	7.47
Hb charge (eq/mol Hb)	−5.6	−5.6	−5.1	−5.6	−8.4
Predicted $\%\Delta F$	0	−6.2	−4.8	+37.6	+28.8

This simulation using our program IONIC-F is for human red blood cells initially suspended in 150 mM NaCl, 5 mM HEPES, 284 mosM, pH 7.4, using the equations previously derived (Freedman and Hoffman, 1979*a*). The symbols for the cell parameters in column 1 are defined as follows: square brackets denote millimolal concentrations; subscript c is intracellular and subscript o is extracellular; C is the total solute concentration (millimolal); ϕ_{Hb} is the osmotic coefficient of Hb; Φ is the osmotic coefficient of the internal or external solution. The isotonic intracellular parameters were taken as follows: 7.3 mmolal [Hb]; 151 mmolal impermeant internal cations; 12.1 mmolal non-Hb cell solutes, denoted by [P] and consisting of 2.0 mmolal ATP, 6.7 mmolal 2,3-diphosphoglycerate, and 3.4 mmolal glutathione; 0.92 osmotic coefficient of non-Hb solutes; −12.5 eq/mol Hb/pH cellular buffering capacity; and 25°C. The predicted initial cell parameters before shrinkage are given in the second column. To simulate water movement alone (third and fifth columns), the equations for osmotic balance and for the osmotic coefficient of Hb (Eqs. A4 and A6 of Freedman and Hoffman, 1979*a*) were combined to yield a cubic equation for the relative water content. To calculate water content in grams per gram of cells for comparison with the data in Fig. 4, Eq. 4 of Freedman and Hoffman (1979*a*) was used with zero extracellular space. The water contents in row 1 are given relative to that of cells in isotonic medium. For shrinkage with NaCl, the osmolality of the medium was raised to 555 mosM by changing the extracellular composition to 300 mM NaCl and 5 mM HEPES, pH 7.4. For shrinkage with sucrose, the extracellular osmolarity was raised to 616 mosM by changing the extracellular composition to 150 mM NaCl, 300 mM sucrose, and 5 mM HEPES, pH 7.4. The predicted changes in the fluorescence of diS-C_3(5) are shown for the calibration estimated in Fig. 6 (2.2 $\%\Delta F$/mV).

outwardly directed Cl concentration gradient (Table III, row 7) and an inside-positive membrane potential (Table III, row 15), neither of which is predicted for shrinkage by NaCl.

With hypertonic sucrose, the Donnan ratio for Cl increases initially (Table III, row 14) because during the efflux of water the intracellular concentration of Cl increases

(Table III, row 7) without a concomitant increase in extracellular Cl; however, with hypertonic NaCl, the Donnan ratio for Cl decreases initially (Table III, row 14) because the osmotically induced increase in the intracellular concentration of Cl (Table III, row 7) is less than the increased extracellular concentration of Cl. Since estimates for red cell Cl conductance (Hunter, 1977; Knauf et al., 1977) are $\sim 10^2$ times that of K or Na (see Beaugé and Lew, 1977, for review), the membrane potential E_m may be equated with E_{Cl}. The model predicts that shrinkage with hypertonic sucrose initially changes the resting potential from −7.0 mV to an inside-positive voltage of +10.1 mV, which then declines to +6.1 mV (Table III, row 15); shrinkage with hypertonic NaCl yields only a small initial hyperpolarization of 2.8 mV with negligible subsequent change (Table III, row 15). These predictions of the nonideal thermodynamic model agree well with the fluorescence traces in Fig. 1, *A* and *B*.

After shrinkage with hypertonic sucrose, the resultant outwardly directed concentration gradient of Cl is dissipated by two electrically silent pathways: exchange with HCO_3 via the Jacobs-Stewart cycle, and HCl cotransport (or Cl/OH exchange). During the initial cell shrinkage and the subsequent efflux of Cl, rapid equilibration of water maintains osmotic equilibrium; thus the predicted ratios of total intracellular to extracellular osmolarities are maintained at unity (Table III, row 13). As anions redistribute, the predicted Cl content decreases from 236 to 191 μeq/g Hb (Table III, row 6), while cation fluxes are undetectable (Fig. 4; Table III, row 4). Since the cyclic flux of protons (or hydroxide) is "osmotically silent" (see Fig. 7), water must leave the cell with Cl to maintain osmotic equilibrium (Table III, row 3). The osmotic pressure, as well as the concentration of charge of the cell cations, hemoglobin, and impermeant nonhemoglobin solutes increase due to the concentrating effect of the loss of water. The decrease in the predicted intracellular Cl concentration of 32 millimolal (Table III, row 7) or 29 mosM is balanced electrically by an equivalent increased negative charge on Hb amounting to 47 millimolal (Table III, row 17), which is partially offset by an increased intracellular cationic charge of 15 millimolal (Table III, row 5). Osmotic balance is achieved as follows: the increased cation concentration corresponds to 14 mosM, impermeant nonhemoglobin solutes rise by 1.2 millimolal (Table III, row 8), or 1 mosM, and Hb rises by 0.8 millimolal (Table III, row 9), which when multiplied by its increased initial and final osmotic coefficients (Table III, row 11) corresponds to an increase of 14 mosM. Thus the predicted effect of the osmotic coefficient of Hb in increasing the osmotic contribution of Hb, and thus of retaining water during equilibration of Cl, is comparable to the osmotic effect of the increased concentration of cations.

The light-scattering experiments indicate that hypertonic sucrose induces an initial rapid decrease in cell volume, followed by an additional slow decrease, in contrast to the stable response seen after shrinkage with NaCl (Fig. 3). The results of the light-scattering experiments are thus also consistent with a net efflux of Cl after shrinkage by sucrose. This finding is also consistent with predictions of the model (Table III, row 2): with only water movement, the water content of cells shrunken with sucrose is 0.497 g/g cells and that of cells shrunken with NaCl is 0.518 g/g cells. As anions redistribute, cells shrunken with sucrose further decrease their water content

to 0.485 g/g cells, predicting an increase in light-scattering intensity, while those shrunken with NaCl increase their water content only slightly to 0.520 g/g cells.

The measurements of cell water and Cl contents before and after shrinkage (Fig. 4) also agree reasonably with the predictions of the nonideal thermodynamic model. The model predicts a ratio of intracellular Cl content after shrinkage to that before shrinkage of 1.04 for shrinkage by NaCl; the measured values in two experiments were 1.03 and 1.05. For shrinkage by sucrose, the corresponding predicted value was 0.82 and the measured values were 0.88 and 0.91. For cell volume, the model predicts a ratio of cell water content (grams per gram of cells) after shrinkage by NaCl to water content before shrinkage of 0.79; the measured value is 0.83 ± 0.01. With sucrose, the corresponding predicted and measured ratios are 0.74 and 0.80 ± 0.01, respectively. The small yet systematic deviations in cell volume at high osmolarities are consistent with those found in previous studies (Freedman and Hoffman, 1979*a*; Solomon et al., 1986).

Quantitative Analysis of Fluorescence

Shrinkage with hypertonic NaCl (which does not result in an appreciable Cl concentration gradient) and shrinkage after pretreatment with valinomycin (so that Cl concentration gradients do not contribute significantly to the membrane potential) both give negligible rates of decay of fluorescence indistinguishable from that of the buffer control. Thus the observed rate of fluorescence decline seen with sucrose (Fig. 1 *A*, upper trace) indicates that the biphasic voltage response requires a transient Cl concentration gradient and a membrane potential dependent on this gradient.

If the initial increase in the fluorescence of diS-$C_3(5)$ after shrinkage with sucrose depends on the Cl concentration gradient, then an increase in the gradient caused by an increase in [sucrose] should result in an increase in the amplitude of the initial fluorescence change, as seen in Fig. 2 *A*. The subsequent decline of fluorescence after shrinkage with sucrose also depends on external [sucrose] (Fig. 2 *B*), as expected if the fluorescence decline is due to dissipation of a Cl concentration gradient created by osmotic perturbation.

Transport of Cl is mediated by capnophorin, a major glycoprotein in the red cell membrane located in band 3 of SDS polyacrylamide gels. DIDS binds to band 3 and inhibits both Cl exchange and Cl conductance (Knauf et al., 1977), but has no effect on water transport. The partial inhibition by DIDS of the initial increase in fluorescence (Fig. 1 *A*, middle trace) is consistent with the reduced anion conductance.

With 300 mM sucrose, the intracellular concentration of Cl immediately after efflux of water, but before any flux of anions, was calculated (Table III, row 7) by modifying the nonideal thermodynamic model to allow for only water movement; the membrane potential was then estimated (Table III, row 15) from the Nernst equation for Cl. The membrane potential was also calculated similarly with [sucrose] varied from 0 to 600 mM. The change in the initial intensity of the fluorescence of diS-$C_3(5)$ measured at varied [sucrose] (Fig. 2) is plotted *vs.* these calculated membrane potentials in Fig. 6. The slope of this plot yields a calibration of 2.2 ± 0.2 $\%\Delta F$/mV at 0.17% HCT. This calibration is consistent with the value of 1.7 $\%\Delta F$/mV previously obtained at a higher HCT of 0.42%, where the dye is less sensitive to voltage

(Freedman and Hoffman, 1979*b*), and is also consistent with a similar value obtained in DIDS-treated cells (Bifano, Novak, and Freedman, 1984). With ethoxzolamide, dissipation of the osmotically induced outward Cl concentration gradient is partially inhibited, thus increasing the fluorescence changes by some 24% and increasing the dye calibration to 2.7 $\%\Delta F$/mV.

A complication in the quantitative analysis of the biphasic response of the fluorescence of diS-$C_3(5)$ to shrinkage with sucrose (Fig. 1 *A*) is that this cyanine dye also responds to changes in intracellular pH as well as to membrane potential (Freedman and Hoffman, 1979*b*). The predicted change in internal pH at equilibrium under these circumstances is 0.22 pH units (Table III, row 16), an amount that would decrease the fluorescence by some 6% (Freedman and Hoffman, 1979*b*), representing ~37% of the measured total decline in fluorescence (Fig. 1 *A*, upper trace). Thus, of the total decline in fluorescence of 16% during 15 min after shrinkage with 300 mM sucrose, ~6% is attributable to increased intracellular pH, ~3% is due to baseline drift, and the remaining 7% is due to the decline of the inside

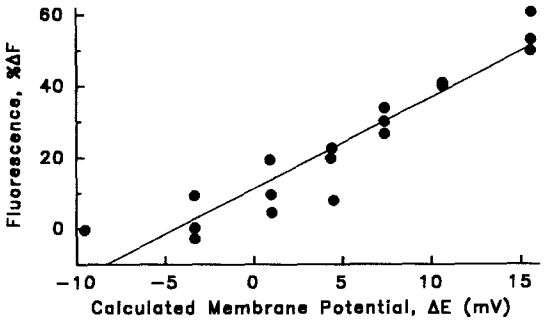

FIGURE 6. Osmotic calibration of diS-$C_3(5)$ fluorescence. The ordinate is the initial change in fluorescence intensity from Fig. 2 *A*. The abscissa is the membrane potential, E_{Cl} (see Table III), as calculated by means of a nonideal thermodynamic model for red cell ionic and osmotic equilibria, assuming that only water leaves the cells upon shrinkage with sucrose. A least-squares linear regression to the data yields a slope of 2.2 ± 0.2 $\%\Delta F$/mV.

positive membrane potential by some 3 mV, in good agreement with the predicted value of 4 mV (Table III, row 15).

Apparent Proton Fluxes

When red cells are shrunken with sucrose under the same conditions in which the biphasic fluorescence response corresponds to a net efflux of Cl, the pH stat experiments reveal an apparent proton efflux of 5.5 μeq/g Hb·min (Table I, row 1). Inhibition of the apparent proton efflux by 10 μM DIDS (Table I, row 4) suggests that transport of protons is associated with that of anions. The magnitude of the apparent proton flux we observe in DIDS-treated cells, 0.4 ± 0.1 μeq/g Hb·min, is in the same range as the values reported by Dissing and Hoffman (1990) at reduced pH.

Pretreatment with valinomycin at 100 mM K_o did not significantly affect the apparent proton efflux induced by sucrose (Table I, rows 2 and 3), whereas the K ionophore did affect the direction of the change in diS-$C_3(5)$ fluorescence and the

polarity of the osmotically induced membrane potential (Fig. 1, *A* and *C*). Addition of valinomycin at the null point of 100 mM $[K]_o$ changed the value of the membrane potential before addition of sucrose only slightly (Fig. 1 *C*), while changing the osmotically induced potential from being dominated by the Cl gradient (Fig. 1 *A*) to being dominated by the K gradient (Fig. 1 *C*). From the changes in diS-$C_3(5)$ fluorescence, using 2.7 $\%\Delta F$/mV as the best estimate for dye calibration under the present experimental conditions, the osmotically induced changes in membrane potential would be +15 mV with ethoxzolamide without valinomycin (Fig. 1 *D*, upper trace) and −9 mV with valinomycin (Fig. 1 *C*). Thus the ethoxzolamide-insensitive apparent proton effluxes (Table I, rows 2 and 3) are insensitive to voltage over a range of 24 mV. Furthermore, the proton flux persisted against an electrochemical gradient (Table II, row 6) and is thus consistent with electrically silent HCl cotransport (or Cl/OH exchange).

The experimental observations are in overall agreement. In the fluorescence (Cl-dependent), pH stat (proton-dependent), light-scattering (volume-dependent), and Cl shift experiments, a net efflux of Cl and water is observed only in cells that have been exposed to hyperosmotic sucrose resulting in a nonequilibrium outward Cl concentration gradient. Dissipation of this gradient is inhibited completely by DIDS and partially by ethoxzolamide. The efflux is absent when cells are shrunken with NaCl, which does not result in an appreciable Cl concentration gradient. Considering that the apparent proton efflux was measured between 1 and 3 min after shrinkage, while the Cl flux is averaged over 15 min, the magnitudes of the apparent efflux of protons, 5.5 μeq/g Hb·min, and the average net efflux of Cl, 1.7 μeq/g Hb·min, are sufficiently close to be consistent with chemical coupling. While better time resolution for the Cl fluxes would be needed to test further the stoichiometric equivalency of the Cl and apparent proton effluxes in the presence and absence of ethoxzolamide, no permeant ionic species other than protons (or hydroxide) are known to be present that could serve as co-ions crossing the membrane at a sufficient rate to balance the anionic charge carried by Cl.

In the presence of bicarbonate and maximally inhibitory ethoxzolamide, it is worthwhile to consider the extent to which the noncatalyzed component of the Jacobs-Stewart cycle might also contribute to an apparent proton efflux (see Fig. 7). For a solution in equilibrium with air, the concentration of bicarbonate (millimolar) is given by $0.03 \cdot P_{CO_2} \cdot 10^{pH-pK}$, where pK for carbonic acid is 6.36 at 25°C. If the air has a dry gas fraction of CO_2 of 0.03%, corresponding to a partial pressure, P_{CO_2}, of 0.23 mmHg at a barometric pressure of 760 mmHg, then the concentration of bicarbonate at an extracellular pH of 7.4 is 76 μM. The rate constant for noncatalyzed dehydration of carbonic acid is $7 \cdot 10^{-3}$ s^{-1} (or 0.42 min^{-1}) at pH 7, and 10 times slower at pH 8 (Magid and Turbeck, 1968). An upper limit for the rate of noncatalyzed dehydration of carbonic acid at the intracellular pH of 7.25 (Table III, row 16) is obtained from the product of the rate constant (0.42 min^{-1}) and the bicarbonate concentration (76 μM), or 32 μeq/liter·min. The measured ethoxzolamide-insensitive apparent proton efflux was 2 μeq/g Hb·min (Table I, row 2), which, with a Hb concentration of 340 g/liter, corresponds to 680 μeq/liter·min. This calculation shows that the noncatalyzed Jacobs-Stewart pathway could only support an apparent proton efflux of $<5\%$ of the osmotically induced flux. Thus at least 95%

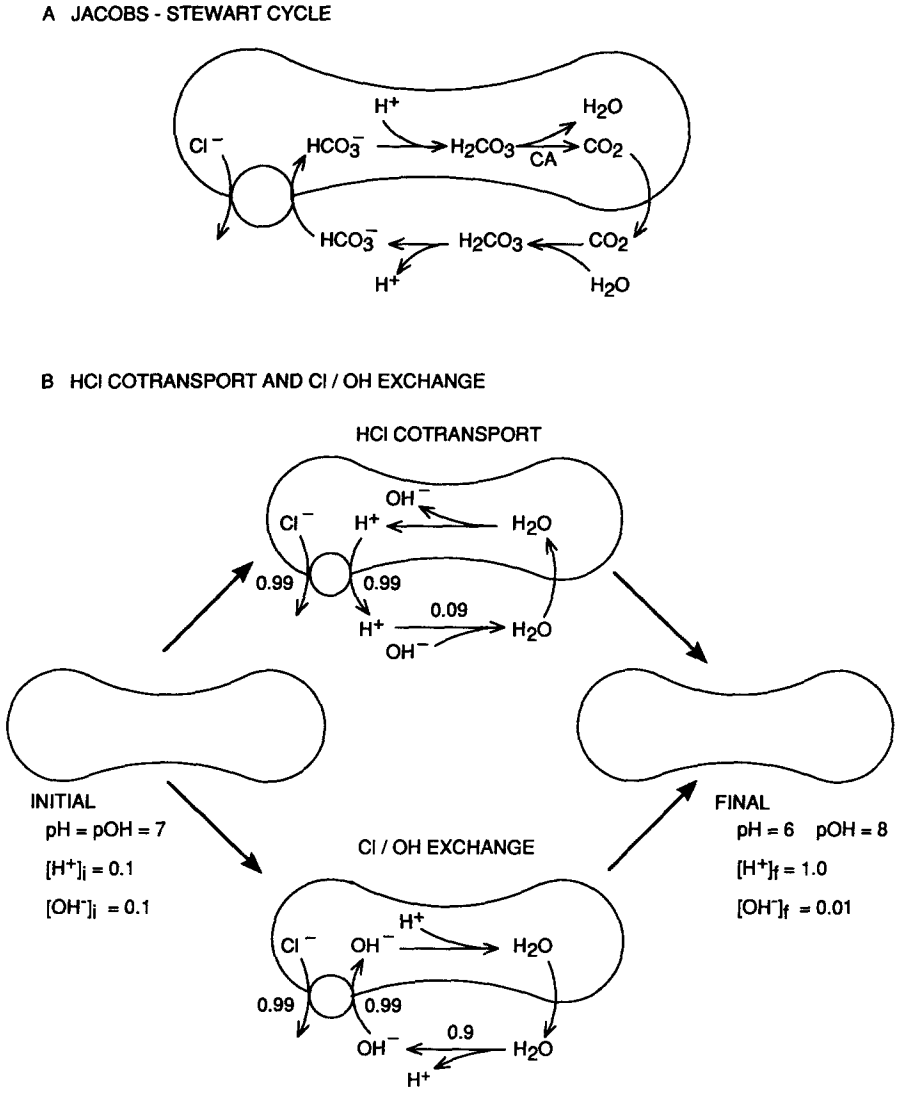

FIGURE 7. Two pathways for apparent proton efflux in human red blood cells. (*A*) The Jacobs-Stewart cycle in which HCO_3^- essentially acts as a carrier for proton efflux. *CA*, carbonic anhydrase that is inhibited by ethoxzolamide. (*B*) Indistinguishability of HCl efflux and Cl/OH exchange. Human red blood cells are schematically represented in unbuffered medium at an initial extracellular pH of 7 (*left diagram*). The middle diagrams represent HCl cotransport (*upper*) or Cl/OH exchange (*lower*) leading to a final extracellular pH of 6 (*right diagram*). The numbers represent micromolar concentrations. The amount of Cl and H or OH crossing the membrane is the same by both mechanisms (see Appendix).

of the osmotically induced apparent proton efflux is attributable to HCl cotransport (or Cl/OH exchange) rather than to Cl/HCO_3 exchange. Another alternative, a direct exchange of Cl for HCO_3, unrelated to a cyclic reaction pathway, would neither dissipate the membrane potential, as seen in Fig. 1, *A* and *D*, nor result in a secondary phase of cell shrinkage, as seen in Fig. 3.

Relationship to Other Studies

Gary-Bobo and Solomon (1968; cf. Solomon et al., 1986) proposed that part of the origin of the so-called "nonosmotic" water in red cells is a dependence of Hb charge on Hb concentration, leading to a Cl shift after cell shrinkage. Evidence for such a Cl shift in the absence of cation movements after shrinkage of red cells with hypertonic salt appeared to support the variable Hb charge hypothesis (Gary-Bobo and Solomon, 1968). However, direct acid-base titrations of Hb in nystatin-treated intact red cells (Freedman and Hoffman, 1979*a*) and of Hb solutions in vitro (Gros, Rullema, Jelkmann, Gros, Bauer, and Moll, 1978) established that the charge on Hb is independent of its concentration. Moreover, the changes in external pH upon shrinkage of red cells in unbuffered media are too small to be consistent with the variable Hb charge hypothesis (Hladky and Rink, 1978), and the proposal was later withdrawn (Solomon et al., 1986). Our experiments do demonstrate net efflux of Cl from red cells after shrinkage with sucrose. The magnitude of the flux we observe ($\sim$7 meq/liter of cell water in 15 min) is much smaller than reported by Gary-Bobo and Solomon (1968). Moreover, our Cl shift is seen only when cells are shrunken with sucrose, and not with NaCl, as was the case in the experiments of Gary-Bobo and Solomon (1968). Thus our results (Fig. 1) confirm that human red blood cells can produce an electrical signal in response to an osmotic stress, albeit under different conditions and by a different mechanism than earlier envisaged.

Movements of proton equivalents and anions across the red cell membrane readily occur in the presence of HCO_3^- and CO_2 via the Jacobs-Stewart cycle (Jacobs and Stewart, 1942). However, even in the absence of HCO_3^- and CO_2, protons can apparently be cotransported with anions. Jennings (1976, 1978) found that an inwardly directed sulfate gradient results in net sulfate/proton cotransport, and also reported that CO_2-independent equilibration of pH is mediated by electrically silent HCl cotransport or OH/Cl exchange. Because the rate of pH equilibration increased in proportion to the gradient of $[H^+]$ rather than of $[OH^-]$, Jennings (1978) favored HCl cotransport over OH/Cl exchange as the transport mechanism. Critz and Crandall (1980) suggested that HCl cotransport occurs at low pH while Cl/OH exchange occurs at high pH. On the basis of the amount of proton equivalents and Cl that cross the membrane, the two mechanisms are indistinguishable (see Appendix). Proton influx into intact red cells (Jennings, 1978; Dissing and Hoffman, 1990) and ghosts (Pitterich and Lawaczeck, 1985) is dependent upon the species of extracellular anion present, and is 97–98% inhibitable by DIDS (Jennings, 1978; Pitterich and Lawaczek, 1985). Gunn (1986) described the kinetics of activation of proton influx by extracellular Cl. Milanick and Gunn (1986) provided kinetic data consistent with the proton binding site on band 3 protein alternatively having access to the cytoplasmic and the extracellular solutions, and with proton transport occurring asynchronously with anion transport. Wieth and Bjerrum (1982) identified titratable transport and

modifier sites on the anion exchanger that might also participate in mediating proton fluxes. Measurements of the binding of the band 3–specific probe NDS-TEMPO indicate that titratable regulator sites are positioned outside the substrate site (Kaufmann, Eberl, and Schnell, 1986). Thus several previous studies have indicated that chemically coupled proton and anion transport occurs in human red blood cells when a proton or anion gradient is established by changing the extracellular anion or proton concentration at constant cell volume, and have begun to assess the role of titratable sites in the mechanism of anion exchange. Our results indicate that HCl cotransport (or Cl/OH exchange) can also occur when an outward Cl concentration gradient is established by increasing the osmotic strength of the extracellular solution.

APPENDIX

The derivation below shows generally and for a specific example that from measurements of net H(OH) and Cl fluxes and changes in extracellular pH it is not possible to distinguish between HCl efflux and Cl/OH exchange in an aqueous suspension of red blood cells. Consider a two-compartment system as illustrated in Fig. 7 *B*, representing cells in unbuffered medium initially at pH 7. Suppose the cells generate protons or consume OH to achieve a final extracellular pH of 6. The change in extracellular proton concentration, $\Delta[H^+]$, is given by

$$\Delta[H^+] = [H^+]_f - [H^+]_i \tag{A1}$$

where f and i represent the final and initial states, respectively. In the example $\Delta[H^+] = 0.9$ μM.

Similarly, the change in extracellular hydroxide concentration, $\Delta[OH^-]$, is given by

$$\Delta[OH^-] = [OH^-]_f - [OH^-]_i \tag{A2}$$

and in the example $\Delta[OH^-] = -0.09$ μM, some 10 times smaller than $\Delta[H^+]$.

From $[H^+]\cdot[OH^-] = K_w$, where K_w is the equilibrium constant for the ionization of water, it follows that $\Delta[H^+]$ and $\Delta[OH^-]$ generally differ from each other,

$$\Delta[OH^-] = -K_w\cdot\Delta[H^+]/([H^+]_f\cdot[H^+]_i) \tag{A3}$$

If bulk electroneutrality is maintained by Cl^- crossing the membrane along with H^+ (or OH^-), then it might appear that a measurement of the net Cl^- flux could distinguish between HCl efflux and Cl/OH exchange. In the example, it appears as though the Cl^- flux would be 10 times smaller for Cl/OH exchange than for HCl efflux. However, the following arguments prove that the net Cl^- flux would actually be the same for the two mechanisms.

Since electroneutrality must be maintained in the extracellular medium,

$$\Delta[Cl^-] + \Delta[OH^-] = \Delta[H^+] \tag{A4}$$

In the example, $\Delta[Cl^-] = \Delta[H^+] - \Delta[OH^-] = 0.99$ μM. In general,

$$\Delta[Cl^-] = \Delta[H^+](1 + K_w/[H^+]_f[H^+]_i) \tag{A5}$$

Thus the change in extracellular $[Cl^-]$, and hence the amount of Cl^- crossing the membrane, depends purely on the final and initial pH, and is independent of whether the mechanism is HCl efflux or Cl/OH exchange.

If the mechanism is HCl efflux, the amount of H^+ crossing the membrane, or $\Delta[H^+]_{mem}$, must equal the amount of Cl^- crossing the membrane, or $\Delta[Cl^-]_{mem}$, and similarly for $\Delta[OH^-]_{mem}$ if

the mechanism is Cl/OH exchange. In the example, $\Delta[H^+]_{mem} = \Delta[OH^-]_{mem} = \Delta[Cl^-]_{mem} =$ 0.99 μM. Thus the same amount of H^+, OH^-, and Cl cross the membrane by either mechanism, even though $\Delta[H^+]$ is 10 times greater than $\Delta[OH^-]$ in the example.

The buffering by water accounts for the apparent disparity. With HCl efflux, 0.99 μM H^+ crosses the membrane, but 0.09 μM combines with OH^- to reduce $[OH^-]$ from 0.10 to 0.01 μM, leaving 0.9 μM H^+ to raise $[H^+]$ from 0.1 to 1.0 μM ($pH_f = 6$). With Cl/OH exchange, 0.99 μM OH^- crosses the membrane while only 0.9 μM of water ionizes, thus producing 0.9 μM of H^+ to raise $[H^+]$ from 0.1 to 1.0 μM, and leaving a deficit of 0.09 μM OH^- to lower $[OH^-]$ from 0.1 to 0.01 μM ($pOH_f = 8$). The only difference between the two mechanisms in the example is the formation of 0.09 μM water during HCl efflux and the ionization of 0.9 μM water during Cl/OH exchange. These small changes in the activity of water would rapidly dissipate due to the high permeability of red blood cells to water. Consequently, it seems impossible to distinguish between HCl efflux and Cl/OH exchange from the measurement of net Cl fluxes and changes in extracellular pH.

This paper is dedicated to A. K. Solomon in appreciation of his extensive studies on red cell transport and osmotic properties.

We thank Aaron S. Freedman for programming assistance, Wilfred D. Stein and Philip B. Dunham for reading and discussing the manuscript, and Larry Stoner for providing us with ethoxzolamide. Part of this research was submitted by J. D. Bisognano in partial fulfillment of the requirements for the M.S. and Ph.D. degrees in the College of Graduate Studies of the State University of New York at Binghamton.

We gratefully acknowledge the support of grants from the American Heart Association (to J. C. Freedman), the New York State Affiliate of the American Heart Association (to J. A. Dix), and the National Institutes of Health (grant GM-28839 to J. C. Freedman). We thank Joseph F. Hoffman for continued interest and discussions, and gratefully acknowledge his grants HL-09906, AM-17433, and GM-00167 for initial support.

Original version received 13 December 1991 and accepted version received 28 January 1993.

REFERENCES

Beaugé, L., and V. L. Lew. 1977. Passive fluxes of sodium and potassium across red cell membranes. *In* Membrane Transport in Red Cells. J. C. Ellory and V. L. Lew, editors. Academic Press, New York. 39–51.

Bifano, E. M., T. S. Novak, and J. C. Freedman. 1984. Relationship between the shape and the membrane potential of human red blood cells. *Journal of Membrane Biology.* 82:1–13.

Bisognano, J. D. 1984. Time course of red cell membrane potential as measured by a fluorescent dye. M.S. thesis. State University of New York at Binghamton, Binghamton, NY. 1–165.

Bisognano, J. D. 1987. Ionic adjustments of membrane potential of red cells under osmotic stress. Ph.D. dissertation. State University of New York at Binghamton, Binghamton, NY. 1–156.

Bisognano, J. D., and J. A. Dix. 1984. Use of a fluorescent probe to monitor the time course of red cell membrane potential after osmotic shock. *Biophysical Journal.* 45:19*a*. (Abstr.)

Bisognano, J. D., J. A. Dix, and J. C. Freedman. 1990. Electro-osmotic transduction by human red blood cells. *Biophysical Journal.* 57:97*a*. (Abstr.)

Critz, A. E., and E. D. Crandall. 1980. pH equilibration in human erythrocyte suspensions. *Journal of Membrane Biology.* 54:81–88.

Dick, D. A. T., and L. M. Lowenstein. 1958. Osmotic equilibria in human erythrocytes studied by immersion refractometry. *Proceedings of the Royal Society of London, Series B. Biological Science.* 148:241–256.

Dissing, S., and J. F. Hoffman. 1990. Anion-coupled Na efflux mediated by the human red blood cell Na/K pump. *Journal of General Physiology.* 96:167–193.

Freedman, J. C., and J. F. Hoffman. 1977. Hemoglobin charge and membrane potentials in human red blood cells at varying volumes. *Proceedings of the International Union of Physiological Sciences.* 13:238.

Freedman, J. C., and J. F. Hoffman. 1979*a*. Ionic and osmotic equilibria of human red cells treated with nystatin. *Journal of General Physiology.* 74:157–185.

Freedman, J. C., and J. F. Hoffman. 1979*b*. The relation between dicarbocyanine dye fluorescence and the membrane potential of human red cells set at varying Donnan equilibria. *Journal of General Physiology.* 74:187–212.

Freedman, J. C., and T. S. Novak. 1989. Optical measurement of membrane potentials of cells, organelles, and vesicles. *Methods in Enzymology.* 172:102–122.

Gary-Bobo, C., and A. K. Solomon. 1968. Properties of hemoglobin solutions in red cells. *Journal of General Physiology.* 52:825–851.

Gros, G., H. S. Rullema, W. Jelkmann, H. Gros, C. Bauer, and W. Moll. 1978. Net charge and oxygen affinity of human hemoglobin are independent of hemoglobin charge. *Journal of General Physiology.* 72:765–773.

Gunn, R. B. 1986. H or OH fluxes activated by inorganic ions on the anion exchanger of human red cells. *Biophysical Journal.* 49:579*a*. (Abstr.)

Hladky, S. B., and T. J. Rink. 1978. Osmotic behavior of human red blood cells: an interpretation in terms of negative intracellular fluid pressure. *Journal of Physiology.* 274:437–446.

Hunter, M. J. 1977. Human erythrocuyte anion permeabilities measured under conditions of net charge transfer. *Journal of Physiology.* 268:35–49.

Jacobs, M. H., and D. R. Stewart. 1942. The role of carbonic anhydrase in certain ionic exchanges involving the erythrocyte. *Journal of General Physiology.* 25:539–552.

Jacobs, M. H., and D. R. Stewart. 1947. Osmotic properties of the erythrocyte. XII. Ionic and osmotic equilibria with a complex external solution. *Journal of Cellular and Comparative Physiology.* 30:79–103.

Jennings, M. L. 1976. Proton fluxes associated with erythrocyte membrane anion exchange. *Journal of Membrane Biology.* 28:187–205.

Jennings, M. L. 1978. Characteristics of carbon dioxide-independent pH equilibration in human red blood cells. *Journal of Membrane Biology.* 40:365–391.

Kaufmann, E., G. Eberl, and K. Schnell. 1986. Characterization of the band 3 substrate in human red cell ghosts by NDS-TEMPO, a sulfonatostilbene spin probe: the function of protons in NDS-TEMPO and substrate-anion binding in relation to anion transport. *Journal of Membrane Biology.* 91:129–146.

Knauf, P. A., G. F. Fuhrmann, S. Rothstein, and A. Rothstein. 1977. The relationship between anion exchange and net anion flow across the human red blood cell membrane. *Journal of General Physiology.* 69:363–386.

Magid, E., and B. O. Turbeck. 1968. The rates of the spontaneous hydration of CO_2 and the reciprocal reaction in neutral aqueous solutions between 0° and 38°. *Biochimica et Biophysica Acta.* 165:515–524.

Milanick, M. A., and R. B. Gunn. 1986. Proton inhibition of chloride exchange: asynchrony of band 3 proton and anion transport sites? *American Journal of Physiology (Cell Physiology).* 250:C955–C969.

Perillie, P. E., and F. H. Epstein. 1963. Sickling phenomenon produced by hypertonic solutions. A possible explanation for the hyposthenuria of sicklemia. *Journal of Clinical Investigation.* 42:570–580.

Pitterich, H., and R. Lawaczeck. 1985. On the water and proton permeabilities across membranes from erythrocyte ghosts. *Biochimica et Biophysica Acta.* 821:233–242.

Ponder, E. 1948. Hemolysis and Related Phenomena. Grune & Stratton Inc., New York.

Savitz, D., V. Sidel, and A. K. Solomon. 1964. Osmotic properties of human red cells. *Journal of General Physiology.* 48:79–91.

Sidel, V. W., and A. K. Solomon. 1957. Entrance of water into human red cells under an osmotic pressure gradient. *Journal of General Physiology.* 41:243–257.

Skoog, D. A., and D. M. West. 1963. Fundamentals of Analytical Chemistry. Holt, Rinehart & Winston, New York. 345–348.

Solomon, A., J. A. Dix, and M. R. Toon. 1986. Osmotic properties of human red cells. *Journal of Membrane Biology.* 91:259–273.

Van Slyke, D. D., H. Wu, and F. C. McLean. 1923. Studies of gas and electrolyte equilibria in the blood. V. Factors controlling the electrolyte and water distribution in the blood. *Journal of Biological Chemistry.* 56:765–849.

Wieth, J. O. 1979. Bicarbonate exchange through the human red cell membrane determined with (^{14}C) bicarbonate. *Journal of Physiology.* 294:521–539.

Wieth, J. O., and P. Bjerrum. 1982. Titration of transport and modifier sites on the red cell anion transport system. *Journal of General Physiology.* 79:253–282.