Hemisodium, a Novel Selective Na Ionophore

Effect on Normal Human Erythrocytes

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ABSTRACT Hemisodium is a novel Na ionophore that belongs to the class of compounds called cryptands. These compounds possess an electron-rich cavity for binding of cations and are conformationally organized during synthesis to favor the selective binding of one cation over another. In media containing 145 mM NaCl and 5 mM KCl, hemisodium (10^{-5} M) increased erythrocyte Na content from 23 to 345 mmol/kg·dry cell solid (dcs) over 4 h and increased water content from 1.8 to 3.5 liter/kg·dcs over the same period. K content decreased somewhat over the same time period, but this fall in K content was prevented entirely by incubation in either low Na media (to prevent net Na entry) or in Cl free media. Thus, the decrease in K content in high NaCl media was due to cell swelling, which activated KCl cotransport, and not due to a direct action of hemisodium on K permeability. Hemisodium-mediated Na transport was conductive, because erythrocyte membrane potential (V_m), determined by diS-C_3-5 fluorescence, changed from -9 to $+22$ mV in high Na media in the presence of hemisodium and DIDS. In cells equilibrated with sulfamate, an anion with low conductive permeability, V_m changed 54 mV per 10-fold change in external Na concentration with the addition of hemisodium. In contrast, a 10-fold change in the external concentration of K, Rb, Cs, or Tl failed to alter V_m in the presence of hemisodium, suggesting a high Na specificity of the ionophore. Na conductance determined from net fluxes increased from 0.04 to 5.2 $\mu S/cm^2$ with 10 μM hemisodium, and with that concentration the ratio of Na to K conductance was 45:1. Among the Na ionophores available so far, hemisodium appears to have the greatest specificity. Hemisodium may be a valuable tool in membrane transport studies.

INTRODUCTION

Ionophores are compounds that facilitate ion transport across cell membranes by forming transportable complexes with ions (carrier mechanism) or by forming pores

within the membrane (channel mechanism). Ionophores have been widely used in membrane transport research to probe mechanisms of channels and carriers in membranes (Mueller and Rudin, 1967; Szabo, Eisenman, and Ciani, 1969; Cass, Finkelstein, and Krespi, 1970), to alter the cation content of a variety of cell types including erythrocytes (Cass and Dalmark, 1973) and cultured HeLa cells (Ikehara, Yamaguchi, Hosokawa, Yonezu, and Miyamato, 1986), and to alter the membrane potential (Kracke and Dunham, 1987).

While highly specific K ionophores (valinomycin and its depsipeptide analogues) are available, the known Na ionophores available at present (nystatin, gramicidin) are somewhat nonspecific and in particular show poor Na-K discrimination (Cass et al., 1970; Myers and Haydon, 1972; Cass and Dalmark, 1973), which limits their usefulness in many applications. Even monensin, a relatively specific Na ionophore, has a Na:K selectivity of no more than 10:1 (Pressman and Fahim, 1982). A highly specific Na ionophore is not available at present.

Hemisodium, whose structure is depicted in Fig. 1, belongs to a class of compounds called hemispherands. Hemispherands were designed and synthesized spe-
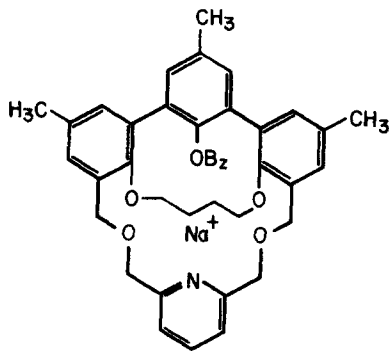

FIGURE 1. Structural formula of hemisodium. Bz represents benzoate.

cifically as host compounds for cations (Cram, 1986). They possess an electron-rich cavity which provides the binding site(s) for the cation of interest. At least half the binding sites of hemispherands have been conformationally organized during synthesis so as to favor the selective binding of one cation over another (Koenig, Lein, Stuckler, Kaneda, and Cram, 1979; Cram, 1986). In the case of hemisodium (Fig. 1), the binding cavity was constricted with the aim of producing a selective increase in Na permeability (Toner, Daniel, Wood, Feltz, and Luss, 1984). While hemisodium has been reported to increase Na selectivity dramatically in thin-film solvent/polymeric membrane electrodes (Battaglia, Chang, and Daniel, 1980), there are no known reports of the effects of hemisodium in biological tissues. Because of the importance of a selective Na ionophore as a tool in membrane transport research, we investigated the effects of hemisodium using intact human erythrocytes as a model for ion transport. Our results suggest that hemisodium is a highly specific Na ionophore.

Portions of this work were published earlier in abstract form (Kaji and Malik, 1990).

MATERIALS AND METHODS

Preparation of Cells

Heparinized blood was collected from four normal volunteers and two patients with sickle cell anemia and processed within 60 min of collection. Blood was centrifuged at 600 g for 10 min and the buffy coat was aspirated. The cells were washed three times in a wash solution containing 150 mM N-methyl glucamine (NMG) Cl, 5 mM dextrose, and 5 mM Tris·HCl, pH 7.4 at 37°C, and packed to a hematocrit of 75–80%.

Intracellular Na, K, and Water Content

Cell electrolytes were measured by flame photometry of lysates from washed cells as described in previous publications (Kaji, 1989, 1990). Water content of fresh cells was measured by drying 0.1 ml of wet cells to a constant weight in an oven at 100°C for 24 h (Kaji, 1989, 1990). Under conditions of rapid cell swelling, the water content of cells was calculated from their mean corpuscular hemoglobin concentration (MCHC) as described (Kaji, 1986).

Anion Equilibration

Cells were equilibrated in media with 150 mM sulfamate substituting for Cl as described by Dunham, Stewart, and Ellory (1980). Cells were first washed in media with 150 mM sulfamate and then incubated three times at 37°C for 60 min in the same media; cells were washed and resuspended between incubations. Finally, cells were washed three times in the same medium at room temperature. Complete anion equilibration was confirmed by the finding of normal cell water content and undetectable cell Cl content; the latter was measured as described previously (Kaji, 1989).

Membrane Potential (V_m)

Calibration. Membrane potential was calibrated by measuring the change in fluorescence of the carbocyanine dye with 1 μM valinomycin in cells at known potentials (Hoffman and Laris, 1974). V_m was modified by varying K gradients in the presence of valinomycin (1 μM) and DIDS (50 μM). Under these conditions, V_m approaches E_K and was assumed to be equal to (RT/F) ln $([K]_i/[K]_o)$, where $[K]_i$ and $[K]_o$ represent intracellular and extracellular K (millimolar), and F, R, and T have their usual meanings. Cells (3 μl) were added to media (2 ml) containing 150 mM (KCl + NMGCl), 5 mM dextrose, 0.05 mM DIDS, 0.2 μM dis-C_3-5, and 5 mM Tris·MOPS, pH 7.4 at 37°C. After reading the fluorescence, valinomycin was added (1 μM final concentration) and the change in fluorescence was recorded 90 s after the addition of the ionophore. The linear relation between the change in fluorescence and V_m in the range of 10–100 mM $[K]_o$ was used to calibrate the V_m for hemisodium studies.

Measurement of V_m with hemisodium. The method for measuring the fluorescence change with hemisodium was the same as above except that (a) 10^{-5} M hemisodium was added instead of valinomycin to initiate the change in V_m, and (b) the media contained 150 mM Cl or sulfamate salts of (Na + NMG). All other conditions including the hematocrit and the dye concentrations were identical to those used for the calibration curve. The change in fluorescence with hemisodium at any given $[Na]_o$ was then converted to V_m using the calibration curve (see above), which was performed on the same batch of cells immediately before this experiment.

Transference Number

The transference number for a cation was calculated as described by Grinstein, Clarke, Dupre, and Rothstein (1982) by using the equation,

$$T_j = (dV_m)/[(RT/F) \ln (C_1/C_2)] \tag{1}$$

where T_j is the transference number for the jth ion, dV_m is the change in V_m on varying the concentration of the jth ion from the initial (C_1) to the final (C_2) value, and R, T, and F have their usual meanings.

Calculation of Relative Permeabilities for Cl and Sulfamate

We calculated the ratio $P_{sulfamate}/P_{Cl}$ by measuring valinomycin-mediated K efflux from erythrocytes equilibrated with Cl or sulfamate (see anion equilibration, above) and suspended at 1% hematocrit in 150 mM NaCl or Na sulfamate. In the presence of 1 μM valinomycin, K efflux is limited only by the permeability of the accompanying anion. Hence, Cl and sulfamate permeability can be approximated from K efflux (Hunter, 1977; Haas, Schmidt, and McManus, 1982; Knauf, Law, and Marchant, 1983). While the calculation of absolute Cl and sulfamate permeability requires constant field assumptions which may or may not be satisfied strictly, the ratio of permeabilities is relatively assumption free.

Alteration of Cell Electrolytes

The nystatin method of Cass and Dalmark (1973) was used to prepare cells with equal Na and K concentrations. Cells were incubated at 4% hematocrit in media containing 75 mM NaCl, 75 mM KCl, 35 mM sucrose, 20 mg/liter nystatin, and 2.5 mM HEPES, pH 7.2 at 4°C for 15 min in the dark. The procedure for washing cells and eluting nystatin has been described in detail previously (Kaji, 1986, 1989). The final cell Na and K concentrations were 73.1 ± 0.9 and 77.3 ± 1.3 mmol/liter cell water, respectively, and the water content was 1.77 liter/kg·dry cell solid (dcs).

Na and K Conductance

Na and K conductances were calculated from the equation of Hodgkin and Huxley (1952),

$$g_M = I_M/(V_m - E_M) = J_M \cdot F/(V_m - E_M) \tag{2}$$

where I_M is the current of ion M (Na or K), J_M is the net flux of monovalent cation in moles per square centimeter per second, F is the Faraday constant, and E_M is the equilibrium potential for the ion. V_m was determined from the change in fluorescence of carbocyanine dye as described above, and E_M was determined from the measured intracellular and extracellular concentrations as $(RT/F) \cdot \ln [M]_o/[M]_i$. Net Na and K effluxes were measured as described below.

Net Na and K efflux. Cells altered with nystatin to contain approximately equal Na and K (see above) were incubated in media with (mM): 10 NaCl, 10 KCl, 130 NMGCl, 5 glucose, 5 Tris·HCl, pH 7.4 at 37°C. The media also contained 0.5 mM ouabain and 0.01 mM bumetanide. Efflux was initiated by adding 10 μM hemisodium. Net Na and K fluxes in millimoles per liter original cells^{-1} per minute^{-1} were calculated from the decrease in cell Na and K content over 3 min. For converting Na and K efflux from millimoles per liter original cells per minute to micromoles per square centimeter per second, 1 liter of original cells was assumed to correspond to 1.75×10^7 cm^2 (Vestgaard-Bogind, Stampe, and Christophersen, 1987).

Zero current Na conductance (g_{Na}^0). g_{Na}^0 was calculated from unidirectional Na fluxes at equilibrium, using the equation of Hodgkin and Keynes (1955). This method has been used to

calculate ionophore-mediated increase in g_K^0 in sheep erythrocytes (Tosteson, Andreoli, Tiffenberg, and Cook, 1968). Intracellular Na and K were 14 and 129 mmol/liter cell water. Therefore, external Na and K were set at 10 and 90 mM so that $E_{Na} = E_K = E_{Cl} = V_m$. The resting V_m of these cells, determined from the fluorescence of diS-C_3-5, was −9.5 mV. Unidirectional ^{22}Na influx was measured by incubating erythrocytes in media containing 10 mM NaCl, 90 mM KCl, 50 NMGCl, 5 mM glucose, and 5 mM Tris·HCl, pH 7.4 at 37°C with a tracer quantity of ^{22}Na and various concentrations of hemisodium for 15 min. Preliminary studies confirmed that the steady-state fluxes were linear up to 20 min with the highest concentrations of hemisodium. Na conductance was calculated as

$$g_{Na}^0 = (F^2/RT)\cdot J_{in} \tag{3}$$

where g_{Na}^0 is the zero current Na conductance in microsiemens per square centimeter, J_{in} is the unidirectional Na influx in micromoles per centimeter^{-2} per second^{-1}, and F, R, and T have their usual meanings.

Water Content of LLC-PK1 Cells

LLC-PK1 cells were purchased from American Type Culture Collection (Rockville, MD) and maintained in Dulbecco's modified Eagle media (DMEM) with 10% fetal bovine serum and 2 mM L-glutamine at 37°C in a 5% CO_2 atmosphere (Amsler and Cook, 1982). Water content was measured by the method of Kletzein, Paiza, Becker, and Potter (1975) from the distribution of the nonmetabolized hexose 3-O-methyl [^{14}C]glucose. Briefly, monolayers of cells grown on plastic Falcon six-well plates were incubated in 2 ml of DMEM containing 1, 2, 5, or 10 mM 3-O-methyl [^{14}C]glucose for 30 min at 37°C. The medium was removed and cells were washed four times with ice-cold phosphate-buffered saline containing 1 mM phloretin, which prevented the efflux of hexose from the cells during washing. The cells were solubilized with 0.2% SDS and cell extracts were counted for ^{14}C radioactivity. Protein content was measured by the fluorescence of tryptophan using method of Avruch and Wallach (1971). Since hexose concentration equalizes in the intracellular and extracellular compartments, the uptake of 3-O-methyl D-glucose gives the intracellular water content, which was expressed as microliters per milligram protein.

Solutions, Chemicals, and Drugs

Hemisodium was obtained from Eastman Kodak Co. (Rochester, NY) (catalog No. 185-2912) and dissolved in a 9:1 mixture of ethanol:DMSO to prepare a 10-mM stock solution. The compound is no longer in stock. The manufacturer has indicated verbally that the company will synthesize the compound if commercial demand for the product develops. All other reagents were from Sigma Chemical Co. (St. Louis, MO), Alfa Products (Ward Hill, MA), or J. T. Baker (through VWR Scientific, Piscataway, NJ). ^{22}Na was obtained from Amersham Corp. (Arlington Heights, IL).

Presentation of Data

Results are presented as means ± standard deviation unless stated otherwise. Because of variation in the absolute rate of K efflux in cells from different donors, the results from different donors have not been pooled. Instead, representative studies are shown in figures. In each case, similar results were obtained in two or more separate studies with cells from different donors. Experimental data points were fitted to equations using nonlinear regression with successive iteration using commercial software packages (Enzfitter; Elsevier-Biosoft, New York, NY, and Sigmaplot; Jandel Scientific, Cortes Madera, CA).

RESULTS

Effect of Hemisodium on Erythrocyte Na, K, and Water Contents

Na content. Hemisodium (10^{-5} M) produced a dramatic increase in intracellular Na content of normal erythrocytes, from 23 to 348 mmol/kg·dcs over a period of 4 h (Fig. 2, closed circles). The hemisodium-induced increase in intracellular Na content was similar in the absence (closed circles) and presence of ouabain (closed squares). The curves for hemisodium and for hemisodium plus ouabain, corrected for zero time values, were well fitted according to the first-order equation $Na_c^t = Na_c^f (1 - e^{-kt})$ (Eq. 4), where Na_c^t and Na_c^f are the Na contents in millimoles per kilogram per dry cell solid at time t and infinity, respectively, and k (min^{-1}) is the rate constant for influx. The computed initial rates of influx were 3.02 mmol/kg·(dcs·min)$^{-1}$ with hemisodium alone and 3.45 mmol/kg·(dcs·min)$^{-1}$ with hemisodium and ouabain.
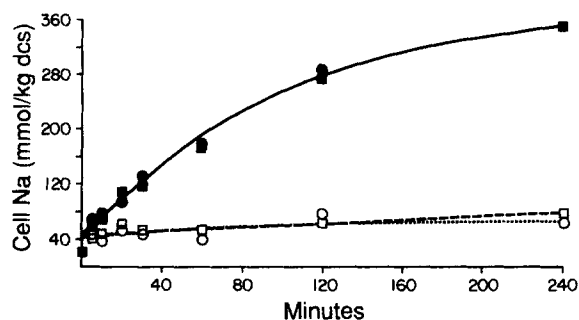

FIGURE 2. Effect of hemisodium (10 μM) with and without ouabain (0.05 mM) on Na content of normal erythrocytes in millimoles per kilogram per dcs over 4 h. Cells were suspended at a hematocrit of 5% in media containing 145 mM NaCl, 5 mM KCl, 5 mM dextrose, and 5 mM Tris·HCl, pH 7.4 at 37°C in the presence of hemisodium (closed circles), hemisodium plus 0.5 mM ouabain (closed squares), no additional reagents (open circles), or 0.5 mM ouabain alone (open squares). The curves for hemisodium and for hemisodium plus ouabain represent the theoretical lines drawn according to the first-order equation (Eq. 4, Results). For hemisodium (closed circles) the initial Na_c was 23 mmol/kg·dcs, the rate constant was 0.0084 ± 0.0013 min^{-1}, and the Na content at equilibrium was 356.2 mmol/(kg·dcs·min)$^{-1}$. For hemisodium plus ouabain, the initial Na_c was 34 mmol/kg·dcs, the rate constant was 0.0098 ± 0.0016 min^{-1}, and the Na_c at equilibrium was 345 mmol/kg·dcs^{-1}.

In control cells incubated in the absence of hemisodium, intracellular Na content increased only slightly, from 23 to 38 mmol/kg·dcs over 4 h in the absence of ouabain (open circles) and from 24 to 54 mmol/kg·dcs in the presence of ouabain (open squares).

K content. K content of normal erythrocytes decreased from 263 to 155 mmol/kg·dcs over 4 h with the addition of 10 μM hemisodium (Fig. 3, closed circles). The decrease in K content was somewhat greater (from 253 to 123 mmol/kg·dcs) in the presence of hemisodium and ouabain (closed squares). The rates of K efflux with hemisodium alone and hemisodium plus ouabain were 0.42 ± 0.04 and 0.53 ± 0.06 mmol/kg·dcs^{-1}·min^{-1}, respectively.

Without hemisodium, cell K content decreased minimally, to 255 without ouabain (open circles) and to 242 with ouabain (open squares) over 4 h.
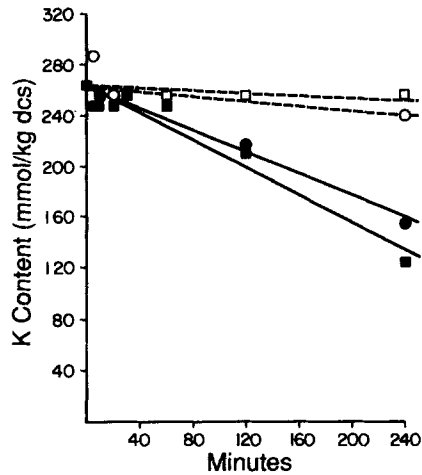
FIGURE 3. The effect of hemisodium (10 μM) with and without ouabain (0.05 mM) K content of normal erythrocytes over 4 h. The composition of the media and the symbols are the same as in Fig. 2. The data points were fitted with linear regressions, because the goodness of the fit did not improve with other, more complex equations. Symbols have the same meanings as in the legend to Fig. 2. For hemisodium, K_C^{init} was 262 mmol/kg·dcs and the efflux rate constant was 0.42 ± 0.04 min^{-1}. For hemisodium plus ouabain, K_C^{init} was 263 mmol/kg·dcs and the efflux rate constant was 0.54 ± 0.06.

While the rate of K efflux with hemisodium was small compared with the rate of Na entry, it was nonetheless important to distinguish whether the increase in K transport was consequent to a direct effect of hemisodium on K permeability, or an indirect consequence of cell swelling which activates KCl cotransport (Kaji, 1986; for review see Dunham, 1989). To distinguish between these possibilities, we examined the effect of hemisodium on erythrocyte K content under conditions designed to minimize cell swelling. To achieve a steady cell volume, external Na concentration was kept low at 10 mM (NMGCl replacement). Intracellular Na concentration was 14 mM in this experiment. Under these conditions, despite a large outwardly directed K concentration gradient ($[K]_i$ = 137 mM, $[K]_o$ = 5 mM), cell K content remained virtually unchanged after 4 h in 150 mM Cl media (252 at 4 h vs. 264 mmol/kg·dcs initial; Fig. 4, triangles). In contrast, the decrease in K content was striking in these
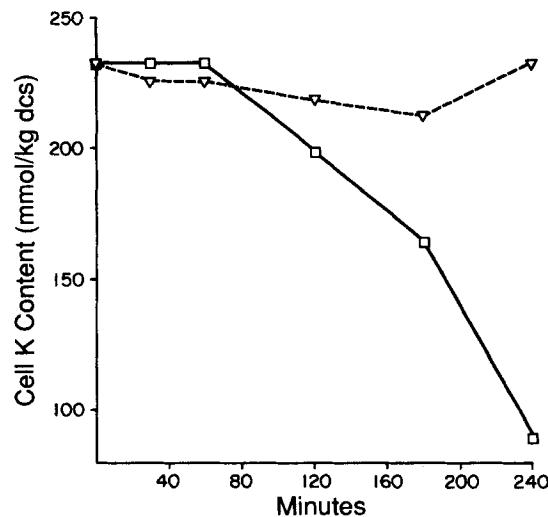

FIGURE 4. Effect of hemisodium on K content (millimoles per kilogram per dcs) in the absence of cell swelling. Cell swelling was prevented by incubating cells in media containing 10 mM NaCl, 135 mM NMGCl, and 5 mM KCl in the presence of 10 μM hemisodium (dashed line, triangles). The control cells (solid line, squares) were incubated in high Na media with hemisodium as described in the legend to Fig. 2 and allowed to swell.

cells when they were swollen by incubation in high (145 mM) Na media (Fig. 4, squares). Thus, the effect of hemisodium on cell K observed in Fig. 3 was secondary to activation of KCl cotransport due to cell swelling and not a direct effect of hemisodium on K permeability. The striking decrease in cell K content was also circumvented in Cl free media (145 Na sulfamate, 5 mM K sulfamate), which also prevents activation of KCl cotransport (274 to 263 mmol/kg·dcs at 60 min; not shown). Cell K content measurements became unreliable in this experiment after 90 min because of severe lysis, which was probably secondary to Na gain in the absence of K loss, leading to greater cell swelling.

Water content. Erythrocyte water content increased from 1.79 to 3.70 liter/kg·dcs with hemisodium and to 3.59 liter/kg·dcs with hemisodium and ouabain (Fig. 5). The curves for hemisodium alone (closed circles) and for hemisodium plus ouabain (closed squares) were fitted with first-order equations similar to those described for

FIGURE 5. Effect of hemisodium (10 μM) with and without ouabain (0.05 mM) on water content of normal erythrocytes over 4 h. For composition of the media and symbols, see the legend to Fig. 2. The curves for hemisodium alone (closed circles) and for hemisodium plus ouabain (closed squares) were fit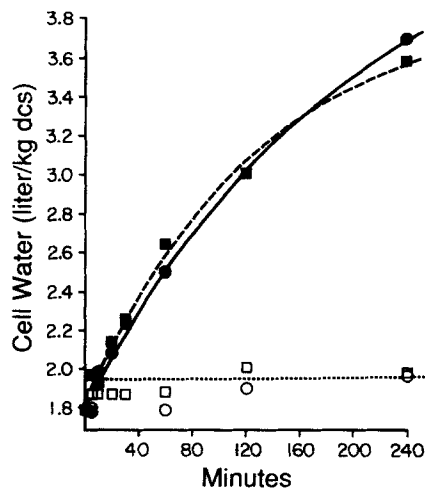ted with first-order equations similar to that described for Na_c (see legend to Fig. 2) and the depicted curves represent theoretical lines drawn according to these equations. For hemisodium, the rate constant was 0.005 min^{-1}, and the water contents at zero time and equilibrium were 1.8 and 4.4 liter/kg·dcs, respectively. For hemisodium plus ouabain, the rate constant was 0.0076 min^{-1}, and the values for W_c at zero time and equilibrium were 1.83 and 3.9 liter/kg·dcs, respectively.

Na_c (see Legend to Fig. 2). The rates of water influx for hemisodium and hemisodium plus ouabain were 0.005 and 0.0076 liter/kg·dcs^{-1}·min^{-1}, respectively.

Without hemisodium, water content increased only slightly over 4 h to 1.96 liter/kg·dcs without and 1.98 liter/kg·dcs with ouabain (Fig. 5, open symbols).

We next examined the correlation between the gain in water content and the net gain in cation content. The gain in water content, which was due to entry of sodium, was attenuated by the simultaneous loss of K. Water content increased in direct proportion to the net gain in (Na + K) content. The concentration of the solution entering the cytoplasm, calculated from the slope of regression line plotting (Na + K) content versus water content, was 167 mmol/liter cell water, which is close to isosmotic (not shown).

Effect of Hemisodium on Na, K, and Water Content of Sickle Cell Erythrocytes

In erythrocytes from two subjects with sickle cell anemia, Na content increased from 58 to 310 mmol/kg·dcs in the first subject and from 28 to 310 mmol/kg·dcs in the second subject with sickle cell anemia (Table I). The decrease in K content was much greater in sickle cell (SS) erythrocytes (Table I). This finding is consistent with earlier reports of greater activity of KCl cotransport in swollen sickle erythrocytes (Brugnara, Bunn, and Tosteson, 1986). As in normal cells, this decrease in erythrocyte K content was abolished in NO_3 media (not shown). The water content of SS erythrocytes increased markedly with hemisodium (1.80 to 2.97 liters/kg·dcs over 4 h; Table I), but this rise was less than that observed in normal erythrocytes, consequent to the greater decrease in K content in SS erythrocytes.

TABLE I

Effect of Hemisodium on SS Erythrocytes Intracellular Content

Minutes	Subject 1			Subject 2	
	Na	K	Water	Na	K
			mmol/kg·dcs		
0	57.9	267.4	1.80	27.9	232
5	61.9	256.2			
20	108.4	246.7			
30	131.6	229.0			
60	178.0	209.9			
120	247.7	164.8			
240	309.6	95.3	2.97	310.1	109

Erythrocyte Na, K, and water contents of SS erythrocytes with 10^{-5} M hemisodium. The media were identical in composition to those described in the legend to Fig. 2. Because of the small quantity of blood obtained from subject 2 with sickle cell anemia, only limited measurements of cation content were possible.

Concentration Dependence

In 145 mM NaCl media, an increase in cell Na content, measured in normal (Hb AA) erythrocytes at 3 h, was observed at hemisodium concentrations as low as 0.2 μM (Fig. 6, left). The effect was progressively greater up to 10 μM, the highest concentration used in this study. Because of extensive cell lysis at 3 h, cell Na and water content at higher hemisodium concentrations could not be measured accurately. Water content also increased progressively with a 3-h exposure to hemisodium and high Na media (Fig. 6, right). Water content was 3.2 liter/kg·dcs at 10 μM hemisodium, compared with a value of 1.74 liter/kg·dcs in control cells not exposed to the ionophore. At 5 h, the Na and water contents were 9 and 6% higher than the corresponding values at 3 h (not shown).

Membrane Potential

To evaluate whether hemisodium-mediated Na flux is electroneutral or electrogenic, we evaluated the V_m of erythrocyte membranes after exposure to hemisodium. Fig. 7 shows the V_m of normal erythrocytes 2 min after the addition of 10 μM hemisodium plotted as a function of log external Na concentration ($[Na]_o$). V_m stabilized at a new
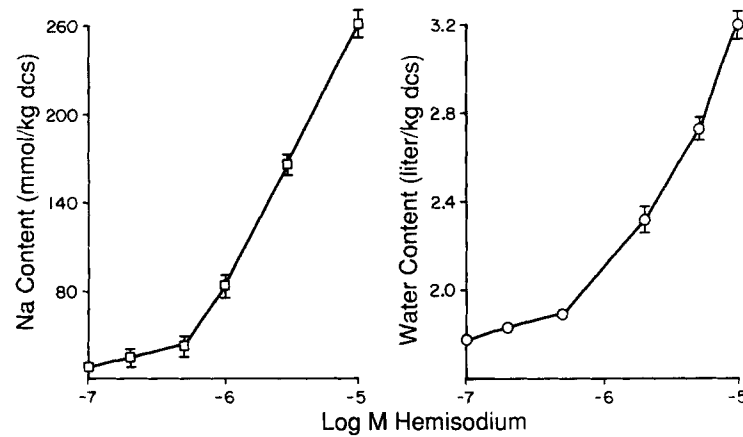
FIGURE 6.   Concentration dependence of cell Na and water contents on hemisodium concentration. Cells were incubated for 3 h in the media described in Fig. 2 in the presence of various concentrations of hemisodium.

value between 90 and 180 s after addition of the ionophore, but some drift in V_m was noted after 3 min. This drift in V_m was most likely consequent to an increase in $[Na]_i$ and a resultant change in E_{Na}. However, at 90 s $[Na]_i$ was not appreciably altered even at the highest $[Na]_o$. The V_m value reported here is the peak value obtained at 90 s.

At a concentration of 10 mM external Na, V_m was unchanged at -10 mV. This was expected from the measured $[Na]_i$ of 14 mM as $E_{Na} = E_{Cl} = V_m$. At other external Na concentrations, V_m increased with increasing $[Na]_o$. The change in V_m was 24 mV per 10-fold change in $[Na]_o$ in Cl media without DIDS (open circles, dashed line). In the presence of DIDS, which inhibits Cl conductance, the change in potential was 36 mV per 10-fold change in $[Na]_o$ (filled circles, solid line). Thus, the transference number
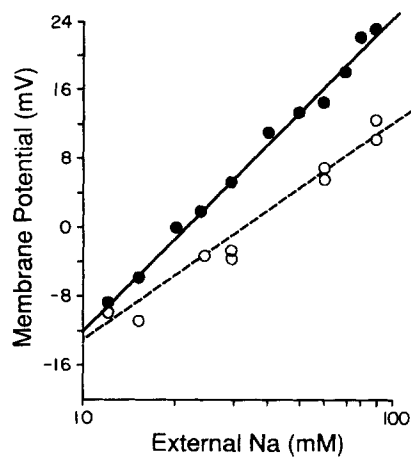

FIGURE 7.   Effect of hemisodium (10 μM) on the V_m of normal erythrocytes in Cl media. Membrane potential versus external Na (plotted on a log scale) in the absence of DIDS (dashed line, open circles) or in the presence of 50 μM DIDS (solid line, closed circles). Media contained 150 mM Cl salts of Na and NMG. The lines depicted are the theoretical lines derived from linear regression of the data points. The equation describing the relation between the V_m in millivolts and log external Na concentration in millimolar was $V_m = 24.2 \pm 1.1 \times \log [Na]_o - 37.8$ in the absence of DIDS and $y = 36.0 \pm 1.2 \times \log [Na]_o - 47.7$ in the presence of DIDS.

(for calculation, see Materials and Methods) for Na with 10 μM hemisodium was 0.39 and 0.60 without and with DIDS, respectively.

While DIDS is an extremely effective inhibitor of electroneutral anion exchange in the erythrocytes, it inhibits conductive Cl permeability only partially, by 50–85% (Knauf et al., 1983; Bennekou and Stampe, 1988). The lack of Nernstian relationship between V_m and [Na]$_o$ may be due to the substantial Cl permeability that persists in the presence of DIDS. Therefore, we evaluated the relationship between V_m and [Na]$_o$ in cells equilibrated with an anion with an extremely low conductive permeability, sulfamate (Payne, Lytle, and McManus, 1990; see Fig. 8). Using valinomycin-mediated K efflux in Na media as a measure of anion permeability (Hunter, 1977; Haas et al., 1982; Knauf et al., 1983), we found that the conductive permeability for sulfamate was very low with a $P_{sulfamate}/P_{Cl}$ ratio of 0.06. The baseline V_m in sulfamate-loaded cells in sulfamate media was −10.3 mV, not different from baseline V_m in Cl media. In sulfamate media with hemisodium, V_m varied by 55 mV per 10-fold change
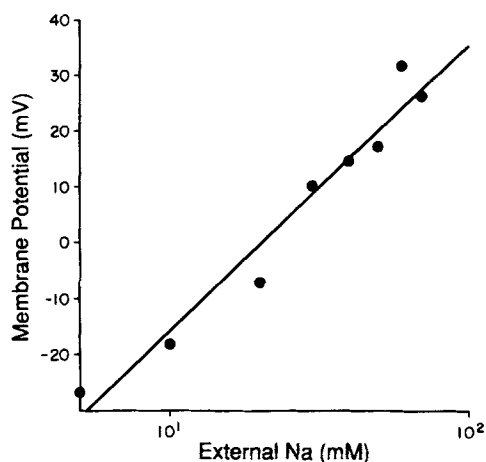

FIGURE 8. Effect of hemisodium (10 μM) on V_m in sulfamate media. Cells were equilibrated in 150 mM sulfamate media as described in Materials and Methods and suspended in media containing 150 mM sulfamate salts of Na plus NMG. Other conditions were the same as in Fig. 7. There was no DIDS in sulfamate media. External Na is plotted on a log scale. The straight line represents the theoretical fit according to the equation $V_m = 55.2 \pm 4.9 \times \log[Na]_o - 73.1$.

in [Na]$_o$, giving a T_{Na} of 0.94. The small difference between the observed T_{Na} (0.94) and ideal T_{Na} (1.0) may be attributable to permeability to sulfamate, OH (or HCO$_3$), or both.

Effect of Hemisodium on Transference Number for Various Cations

We next investigated the hemisodium-mediated increase in the transference number for various monovalent cations. Cells were incubated in media containing either 10 or 100 mM Li, K, Rb, Cs, or Tl, with NMG replacement. Hemisodium (0.1 mM) was added and the V_m was recorded. Table II shows that there was little or no change in V_m with Li, K, Rb, Cs, or Tl. The transference number was 0.6 with Na and 0.03 or less with all other cations. Thus, hemisodium selectively increased the conductive permeability for Na.

TABLE II

Effect of Hemisodium on the Transference Number of Various Cations

Cation (mM)	Transference number	
	Baseline	With hemisodium (10 μM)
Na	0.01	0.60
Li	0.02	0.03
K	0.01	0.01
Rb	0.01	0.01
Cs	0.001	0.003
Tl	0.001	0.002

Membrane potentials were measured in the presence and absence of 10 μM hemisodium. When $[Na]_o$ was varied, NMGCl replaced Na such that NaCl + NMGCl = 150 mM. When other cations were used, media contained 10 mM NaCl, 10 or 100 mM XCl, and 130 or 40 mM NMGCl. All media contained 5 mM dextrose, 50 μM DIDS, and 5 mM Tris.MOPS, pH 7.4 at 25°C. X represents monovalent cation other than Na. The transference number was calculated as described in Materials and Methods.

Effect of Hemisodium on Na Conductance

To measure Na and K conductance, cells were altered with nystatin to contain 75 mM each of Na and K. These cells were incubated in media containing 10 mM NaCl, 10 mM KCl, and 130 mM NMGCl. Hemisodium was added in various concentrations from 0.1 to 100 μM and the change in V_m was monitored by the change in fluorescence of diS-C_3-5 as described above.

Fig. 9 shows the change in V_m as a function of hemisodium concentration. As Na-loaded cells were suspended in media with low $[Na]_o$, E_{Na} was −53 mV. With increasing hemisodium concentration, V_m approached E_{Na} and hyperpolarized progressively. Because of these changes in V_m, the driving force V_m-E_{Na} decreased with increasing hemisodium concentration. Because V_m was always inside negative (−9 to −37 mV) in this experiment (Fig. 9), the Na and K effluxes measured in parallel experiments (see below) could not be attributed to the voltage-activated, nonspecific cation channel which opens only in cells with inside positive V_m (Halperin, Brugnara, Tosteson, and Tosteson, 1989).
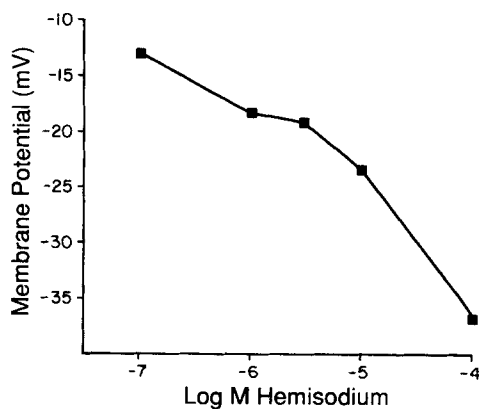
FIGURE 9. Effect of various concentrations of hemisodium on V_m. Cells were altered with nystatin to contain (in millimoles per liter cell water) 73 Na and 76 K, and suspended in media containing 10 mM NaCl, 10 mM KCl, 130 mM NMGCl, 5 mM glucose, 0.5 mM ouabain, 0.01 mM bumetanide, and 5 mM Tris·HCl, pH 7.4 at 37°C. Hemisodium (10 μM) was added at time 0 and V_m was recorded at 90 s. Unlike studies described in Fig. 7, V_m was stable between 90 and 180 s.

Fig. 10 shows the net Na and K efflux from the same batch of cells at the same hemisodium concentrations. Because hemisodium altered V_m, and because the other Na transport pathways (Na-K pump, NaK2Cl cotransport) were inhibited (by adding ouabain and bumetanide), we assumed that net Na and K fluxes in the presence of hemisodium were conductive, and we calculated Na and K currents (I_{Na} and I_K in nanoamperes per square centimeter) from these fluxes as $J_{Na} \cdot F$ or $J_K \cdot F$, where J_{Na} and J_K are the Na and K effluxes in moles/(square centimeter·second). Despite increasing hyperpolarization, which would tend to retard Na efflux, Na efflux increased with increasing hemisodium concentration (Fig. 10), suggesting that Na conductance must have increased with increasing hemisodium concentration. In contrast, K efflux did not increase appreciably with hemisodium (Fig. 10).

Na and K conductances were calculated from the net Na or K currents (calculated from net fluxes; see Materials and Methods) and the driving force (V_m-E_M), where E_M is the equilibrium potential for Na or K. Fig. 11 shows that Na conductance was a
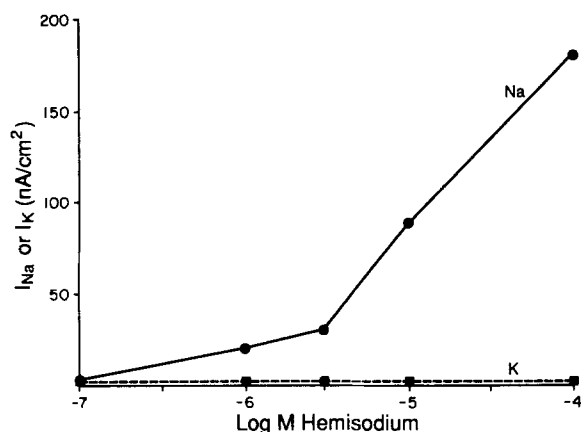

FIGURE 10. Effect of hemisodium on Na and K currents. Cells with equal Na and K concentrations (see legend to Fig. 9) were suspended in 10 mM Na and 10 mM K media with 0.5 mM ouabain and 0.01 mM bumetanide (Fig. 9 for composition). Hemisodium (10 μM) was added at time 0. Net Na and K efflux was linear over 3 min and measured as millimoles per liter original cells. Na and K currents were calculated from cation fluxes assuming that fluxes were conductive.

linear function of hemisodium concentration on a double logarithmic plot and increased sharply with hemisodium concentration, whereas K conductance was not similarly affected. At 100 μM hemisodium, the ratio of Na to K conductance was 45:1, confirming that hemisodium exhibits a high Na/K discrimination.

Zero Current Na Conductance

Zero current Na conductance (g^0_{Na}) was calculated from unidirectional Na fluxes under equilibrium conditions so that $E_{Na} = E_{Cl} = E_K = V_m$ (see Materials and Methods and legend to Fig. 12 for details). Without hemisodium, g^0_{Na} was 0.25 μS/cm². With hemisodium, there was a progressive increase in g^0_{Na}. At 100 μM hemisodium, zero current Na conductance reached a value of 6.44 μS/cm². When the log of g^0_{Na} was plotted versus hemisodium concentration (Fig. 12), the plot was linear in the range between 10^{-7} and 10^{-4} M hemisodium and showed a slope of 0.54 ± 0.05.
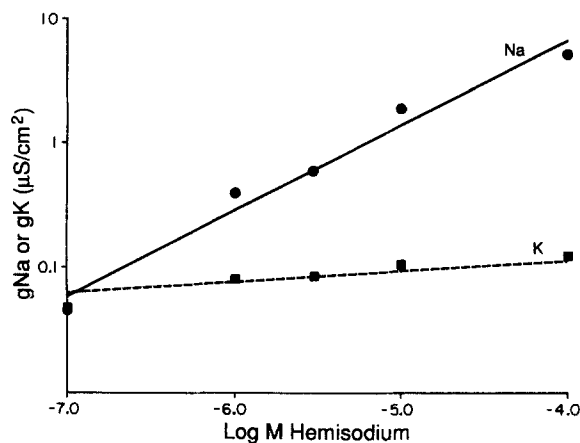
FIGURE 11. Na and K conductances as a function of hemisodium concentration on a logarithmic plot. g_{Na} and g_K were calculated from net Na and K efflux (Fig. 10), V_m (estimated as described in Fig. 9), and E_{Na} or E_K. The equation for Na conductance was log g_{Na} = 0.5 ± 0.04 × log M hemisodium + 3.6. The line for g_K was fitted by eye and has no connotation.

Effect of Hemisodium on Renal Tubular Cells

To evaluate whether the effects of hemisodium were confined to erythrocytes or could also be seen in epithelial cells, we examined the effect of 10 μM hemisodium on the water content of LLC-PK1 cells, a cultured renal tubular cell with characteristics of the S2 segment of the proximal tubule. In three separate experiments, hemisodium for 30 min markedly increased the cell water content of LLC-PK1 cells (Table III). While cell Na was not measured because of technical problems, the increase in water content suggests that cell Na content was increased.

DISCUSSION

The lipid layer of cell membranes forms an effective energy barrier to the passage of cations. Ionophores that act as ion carriers or form ion channels reduce this energy
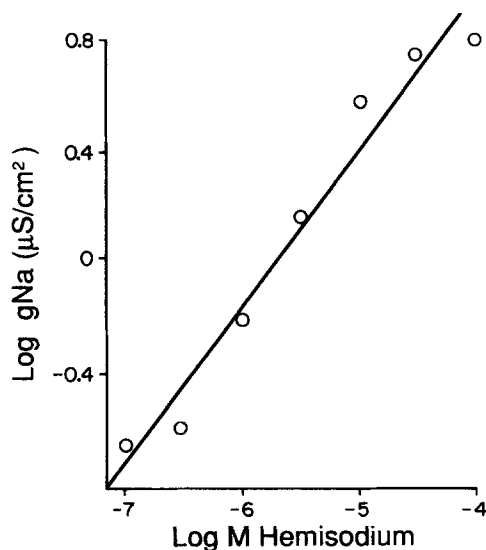

FIGURE 12. Zero current Na conductance as a function of hemisodium concentration. Unidirectional Na influx was measured in in media containing (mM) 10 NaCl, 90 KCl, 50 NMGCl, 0.5 ouabain, and 0.02 bumetanide. The measured intracellular Na and K concentrations were 14 and 141 mM, respectively, and the measured V_m was −9 mV. Zero current Na conductance was calculated from unidirectional fluxes as described in Materials and Methods.

barrier and facilitate the transport of small ions across cell membrane. The study of membrane transport has been greatly facilitated by ionophores (Pressman, 1976). The introduction of hemisodium, a selective Na ionophore, fills a void in the repertoire of tools available to probe transport phenomena.

When hemisodium is added to a suspension of erythrocytes in high Na media (Fig. 2), the resulting Na and K fluxes are mediated by at least three different pathways: (a) the hemisodium-mediated increase in Na permeability, (b) the activation of KCl cotransport, and (c) the activation of a nonspecific cation channel which is irreversibly activated with inside positive potentials (Halperin et al., 1989). Although membrane potentials were not measured pari passu with flux measurements shown in Fig. 2, it appears highly likely from the studies shown in Fig. 7 that the membrane potential of cells in high Na media turned inside positive with the addition of hemisodium. Thus, Figs. 2 and 6 provide a reliable representation of the expected changes in Na content upon addition of hemisodium in high Na media, but should not be used to estimate the direct effect of hemisodium on Na transport.

Ionophore-induced increase in cation permeability may be electrically silent (if the ionophore promotes the exchange of cation for protons or for another cation) or

TABLE III

Effect of Hemisodium on Water Content in LLC-PK1 Cells

	Water content	
Experiment	Baseline	Hemisodium (10 μM)
	μl/mg·protein	
1	7.5	11.4
2	7.7	10.5
3	7.2	12.8

conductive in nature. The change in V_m with hemisodium upon changing external Na suggests that hemisodium-induced Na transport is conductive.

Na conductance with hemisodium was measured under conditions designed to maintain an inside negative V_m and thereby avoid the activation of the voltage-gated cation channel (Figs. 9–11). The finding that hemisodium increased Na conductance nearly 40-fold suggests that hemisodium has a direct effect on Na permeability. The ratio of Na to K conductance (with identical Na and K concentrations) was nearly 50-fold, confirming the specificity of the hemisodium effect on the intact cell membrane.

The relationship between ion conductance and the power of ionophore concentration has been used to derive the number of ionophore molecules forming a single transport unit (Cass et al., 1970). In our studies, the slope of the log plot of zero current Na conductance as a function of aqueous hemisodium concentration was 0.5 (Fig. 12). A slope of less than one is difficult to interpret mechanistically in terms of ionophore:ion stoichiometry. One possible explanation for our finding is the saturation of the ionophore in the membrane phase at higher aqueous concentrations. Thus, the slope of Na conductance versus membrane hemisodium concentration may be one (or more), and the slope of less than unity for the aqueous hemisodium

concentration may be explained by postulating that membrane hemisodium concentration increased less steeply than the aqueous ionophore concentration.

While the precise number of hemisodium molecules forming a single transport unit remains unknown, it is likely to be small. The hemisodium molecule (Fig. 1) appears too small to span the membrane thickness, even with a dimer or a tetramer. Thus, while the possibility that many hemisodium molecules stack together to form a pore cannot be completely excluded, it is more likely that hemisodium acts as a carrier, and combines with Na ion to solubilize it into the hydrocarbon interior of the membrane.

Several lines of evidence suggest that hemisodium increases Na permeability with a greater selectivity compared with other Na ionophores available to date. In thin film solvent polymeric membrane electrodes, hemisodium showed a Na/K selectivity ratio of 167:1 (Toner et al., 1984). In these studies, Na gain was accompanied by little or no K loss despite large K gradients when activation of KCl cotransport was prevented by using sulfamate media. At a concentration of 100 μM hemisodium, the ratio of Na/K conductance was 45:1 (Fig. 11). Finally, in the presence of hemisodium, erythrocyte V_m was not sensitive to large changes in concentrations of external cations other than Na (Table II). Taken together, these findings suggest that hemisodium is highly selective in its action on the Na ion compared with any other ionophore at the present time.

Hemisodium was initially synthesized for technological applications, with the intention of using hemispherand doped membranes as ion-selective electrodes. However, hemisodium appears to have exciting biological and scientific applications over and above any technological application. Thus, hemisodium may be useful in altering intracellular Na concentrations and especially in preparing Na free cells, a task that is cumbersome with nystatin. In addition, low concentrations of hemisodium in the presence of Cl free media may be used for obtaining cells with the desired degree of cell swelling without alteration of intracellular K concentration. An important use for hemisodium may be to shift V_m away from E_{Cl}. In many cell types, Cl is distributed at thermodynamic equilibrium, so that $V_m = E_{Cl}$. A change in the Cl ratio in these cells changes both V_m and the electrochemical Cl potential. Therefore, it becomes nearly impossible to elucidate the mechanism of anion-coupled cation transport unless V_m is shifted away from E_{Cl} (Haas et al., 1982). Shifting V_m away from E_{Cl} by valinomycin is not a good strategy in many cases, because valinomycin-mediated K flux swamps other K fluxes under examination (i.e., Cl-dependent K transport) and renders measurements of anion-dependent K transport impracticable. In these cases, hemisodium may be used to shift V_m away from E_{Cl} and dissect the mechanism of coupled transport. Hemisodium may also be used to probe the dependence of many electrogenic coupled transport processes on membrane potential. The finding that hemisodium was an effective ionophore in cultured renal epithelial cells widens the scope of applications for this compound as a research tool. The large gain in cell water suggests a possible use for hemisodium in diluting hemoglobin concentration to prevent sickling of erythrocytes, but its effect on other (epithelial) cells may preclude this use. It is likely that hemisodium will find many more applications as it becomes widely known.

In summary, we have described the effects of a novel Na ionophore, hemisodium,

on the cation and water contents and membrane potential of intact normal human erythrocytes. The ionophore appears to exhibit the highest Na selectivity among the Na ionophores available at present. This novel Na ionophore may be a very valuable probe in the study of transport phenomena in general and Na transport in particular.

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