Sulfate Flux in High Sodium Cat Red Cells

R. I. SHA'AFI and E. PASCOE

From the Department of Physiology, School of Medicine, and the School of Agricultural Sciences, American University of Beirut, Beirut, Lebanon

ABSTRACT The transport of radioactive sulfate in cat red cells has been studied. The rate constant for $^{35}SO_4$ inward movement under steady-state conditions is 0.24 ± 0.02/hr. This movement was found to be sensitive to osmotic changes in cell volume and to the nature of anions in the incubation medium; it increases with increasing cell volume and decreases with decreasing cell volume. The anions SCN, NO_3, and I were found to inhibit the uptake of $^{35}SO_4$. Furthermore, 1-fluoro-2,4-dinitrobenzene at a concentration of 1 mM inhibits (>90%) this uptake. The inward movement of erythritol-^{14}C shows qualitatively the same dependence on cell volume as $^{35}SO_4$, but it is insensitive to the nature of the anion present in the bathing medium. It was also found that the usually observed inhibition of radioactive Na uptake by SCN in cat red cells can be reversed when cell volume is increased.

INTRODUCTION

Mammalian red cells of most species investigated maintain significant concentration gradients of Na and K across their plasma membranes by means of an Na-K coupled pump (1–4). This pump is driven by metabolic energy derived from hydrolysis of adenosine triphosphate (ATP) by membrane-associated ATPase. However, in cat and dog red cells the cellular concentrations of Na and K approach those of the plasma (5). In addition, the cation transport mechanism(s) in these cells does not conform to the pattern found in human red cells (2, 6–8). For example, there is no measurable ouabain-sensitive component of either K influx or Na efflux. Of particular interest is the dependence of fluxes of these ions on cell volume, as well as on the nature of anions in the bathing medium. K influx is highest when the cells are swollen and lowest when the cells are shrunken, whereas the situation is reversed for Na (7, 8). In cat red cells, substitution of thiocyanate, SCN, for chloride in the bathing medium increases K influx and decreases Na influx and efflux (6), whereas in human red cells both influxes are accelerated (9).

Transport in cat red cells of an anion, sulfate, under various experimental conditions is the subject of the present study.

MATERIALS AND METHODS

Blood was withdrawn from anesthetized cats into a heparinized syringe (10,000 units/ml, 4 ml/liter) by heart puncture. The blood was centrifuged for 15 min at 1500 *g* and the plasma and buffy coat were carefully removed. The red cells were then washed three times in 4 vol of incubation medium whose composition has been reported elsewhere (8). This solution contained 1 mM nonlabeled SO_4 or erythritol to act as carrier. The washed red cells were resuspended in the incubation medium at 10% hematocrit and were incubated for 30–60 min in a water-bath shaker at 38°C before tracer was added. After addition of the tracer, the suspension was thoroughly mixed for approximately 60 sec and then samples were removed for hematocrit determinations and medium radioactivity analysis. At specified intervals thereafter, samples of the suspension were removed. The erythrocytes were separated from the labeled medium by centrifugation and washed three times in ice-cold nonlabeled medium. The amount of radioactive sulfate lost during the washing procedure at 38°C is less than 5%. Cooling the washing solution decreases the amount lost. Cells were always recovered by centrifugation. The packed red cells were then hemolyzed with distilled water (1 ml packed cells to 20 ml water). A known volume of this hemolysate was taken for hemoglobin measurement and another for isotope counting.

The samples were prepared for counting as follows. Known fractions, usually 0.5 ml of the hemolysate, were mixed with an identical volume of 0.5 M perchloric acid and the suspension was centrifuged. A known volume of the clear supernatant was pipetted into a counting vial containing Bray's solution, and excess acidity was neutralized by addition of 0.05 ml of 1 N NaOH. Counting was done in a Packard Tri-Carb liquid scintillation counter (Packard Instrument Co., Downers Grove, Ill.). $^{35}SO_4$ in aqueous solution was obtained from The Radiochemical Centre, Amersham, England, as was erythritol-^{14}C.

Hemoglobin was determined from the optical density at 540 mμ after conversion of hemoglobin to cyanmethemoglobin. The osmolarity was measured by means of a Fiske Osmometer (Osmometer Model G-62, Fiske Associates, Inc., Bethel, Conn.).

Solutions of 1-fluoro-2,4-dinitrobenzene (Eastman Organic Chemicals, Rochester, N. Y.) were prepared in 25% methanol. Final methanol concentration in the incubation medium was 2.5%.

Steady-state unidirectional sulfate influx was calculated according to the kinetics of a two-compartment system (10, 11). Errors stated in this paper represent 1.0 SE of the mean.

RESULTS AND DISCUSSION

Steady-State Sulfate Flux

In these experiments, the washed cells were incubated for approximately 4 hr at 38°C with the suspension medium containing 1 mM nonlabeled SO_4. This was sufficient time to reach equilibrium. The experimentally determined distribution of SO_4 across the membrane, which was done by determining the partition of $^{35}SO_4$ between red cell water and the incubation medium,

was within 5% of the value calculated from the chloride distribution ratio. The results of a typical experiment on $^{35}SO_4$ influx are shown in Fig. 1. It appears from the figure that there is only one suitable rate constant. Table I summarizes the values for sulfate rate constant found in 11 experiments. The average value for the rate constant in cat red cells, 0.24/hr, is almost one order of magnitude lower than the corresponding value of 2.207/hr for human red cells under similar experimental conditions reported by Wieth (10).

In mammalian red cells, the high anion permeability (12, 13) is generally attributed to the presence of positive, fixed charges lining "pores" which are assumed to be located in the cell membrane. These charges act as a barrier

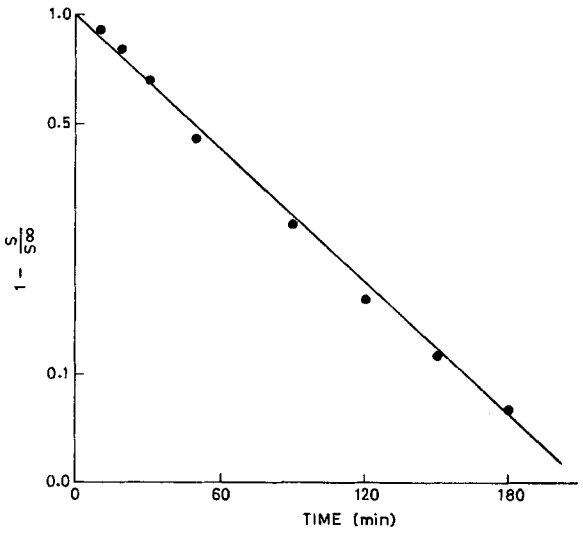

FIGURE 1. Time-course of $^{35}SO_4$ uptake in cat red blood cells. S and $S\ \infty$ refer to the specific activity of $^{35}SO_4$ in the cell at $t = t$ and $t = \infty$, respectively.

to cation diffusion and also as an accelerant for anion passage. The low value of the $^{35}SO_4$ rate constant in cat red cells can be due to either (*a*) a decrease in the number of these charges, or (*b*) the same number of charges but a decrease in the effective charge density. This decrease can be the result of an increase in pore size, a change in the location of these charges, or a change in the environment surrounding the fixed charges. Any of these factors will greatly influence the strength of the interaction between the membrane and the permeating molecule. A decrease in effective charge density will also result in an increase in the ouabain-insensitive components of Na and K fluxes and also an increase in divalent cation movement. It is known that Na and K fluxes in cat red cells are greater than the corresponding ouabain-insensitive components in human red cells (6), and ^{28}Mg inward movement is one order of magnitude higher in the former than in the latter cells (14). There are two

TABLE I
SULFATE RATE CONSTANT IN CAT RED CELL

Exp. No.	Rate constant
	hr^{-1}
1	0.26
2	0.24
3	0.20
4	0.25
5	0.21
6	0.23
7	0.26
8	0.22
9	0.23
10	0.25
11	0.23
Average	0.24
SE	0.02

lines of evidence which may be taken to support the idea of larger pore size. First, osmotic water permeability is considerably higher in cat than in human red cells (15). Second, in cat red cells the permeability to Na, P_{Na}, is higher than the permeability to K, P_K.

Effect of Cell Volume on $^{35}SO_4$ Rate Constant

A phenomenon of particular interest in cat and dog red cells is the dependence of univalent cation fluxes on cellular volume (2, 8). Whether or not anion influx is also dependent on cellular volume was investigated with $^{35}SO_4$. The cellular volume was changed by altering the NaCl concentration alone. The relative cell volume was calculated from the relation (16):

$$V/V_o = 1 + 0.72(T_o/T - 1)$$

in which V_o and V are the cell volume at 290 mosmole/liter and any test osmolarity, respectively, and T_o and T are the osmolarity of isosmotic solution (290) and the test solution. Table II summarizes the results. It is quite clear from the table that the rate constant is highest in swollen cells and lowest in shrunken cells. This dependence is similar to that observed for K influx and opposite to that for Na influx in these red cells (2, 8).

Dependence of $^{35}SO_4$ movement on cellular volume is consistent with the view that osmotic alterations of cell volume lead ultimately, either directly or through a change in the concentration of some intermediate metabolite(s), to conformational changes in the membrane. Structurally, changes could occur in both steric and charge characteristics of the paths used by hydro-

philic solutes. If indeed there were steric changes, then one could predict sensitivity of movement of certain uncharged hydrophilic solutes to osmotic changes in cell volume. Furthermore, this sensitivity would be less than that of charged solutes if in addition to steric changes there are also changes in charge characteristics of these paths. This prediction is borne out by experimental results. In cat red cells the uptake of erythritol-^{14}C was found to be dependent on cell volume, but not to the same degree as was $^{35}SO_4$ uptake.

TABLE II

DEPENDENCE OF SULFATE RATE CONSTANT ON CELL VOLUME

Exp. No.	Relative cell volume	Rate constant	Relative rate constant
		hr^{-1}	
1	0.84	0.17	0.7
	1.00	0.25	1.0
	1.06	0.47	1.9
	1.24	1.12	4.5
2	1.00	0.21	1.0
	1.19	0.84	4.0
	1.31	3.99	19.0
3	1.00	0.24*	1.0
	1.06	0.57	2.4
	1.15	1.19	5.0
4	0.81	0.12	0.5
	0.84	0.15	0.6
	0.92	0.23	0.9
	1.00	0.25	1.0
	1.03	0.32	1.3
5	1.00	0.20	1.0
	1.02	0.26	1.3

* This is the average value taken from Table I.

These results are shown in Fig. 2. The uptake is highest in swollen cells and smallest in shrunken cells. For comparison, the results given in Table II are plotted on the same graph.

Such interdependence of solute movement and cell volume suggests two possible natures for the steric changes occurring: (*a*) osmotic swelling of these red cells results in an apparent increase in physical size of the path(s) used by these solutes, or (*b*) osmotic swelling produces an increase, and shrinkage a decrease, in the hydrated state of the membrane. An increase in hydration will increase the solubility of uncharged hydrophilic solutes in the membrane and therefore increase their rate of movement. Either of these two possibilities

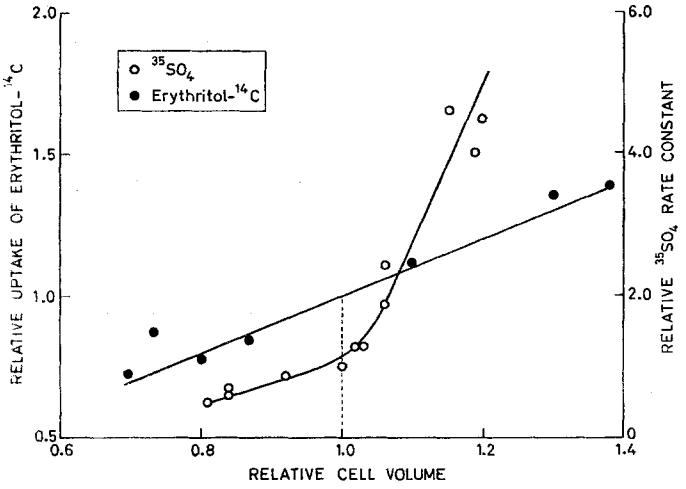

FIGURE 2. Variation of erythritol-^{14}C and $^{35}SO_4$ uptake with cat red cell volume.

suffices to explain the observations of Rich et al. that water filtration permeability increases with decreasing medium osmolality (swollen cells), and that this permeability is much more dependent on medium osmolality in dog than in human red cells (17). Inconsistent with these two possibilities, however, is the observation that Na movement is lowest in swollen and highest in shrunken cells.

Effect of 1-Fluoro-2,4-Dinitrobenzene on $^{35}SO_4$ Rate Constant

Berg et al. (18) showed that 1-fluoro-2,4-dinitrobenzene (DNFB) induces a dramatic increase in Na and K influxes in human red cells. They attributed this effect to abolition of the positive charges of amino groups in the membrane. Recently, Passow put forth arguments to rule out the possibility that DNFB produces its effect by interacting with other groups such as SH, phenolic OH, and imidazole (13). In cat red cells, DNFB was found to lower significantly the rate constant of inward movement of $^{35}SO_4$; Fig. 3 shows a dose-response curve and Fig. 4 shows the kinetics of the onset of inhibition. One striking characteristic of the inhibitory action of DNFB on $^{35}SO_4$ influx is the extreme sensitivity of the cat compared to the human red cell. Under apparently similar experimental conditions, DNFB produces its effect in cat red cells at a concentration one order of magnitude lower than that needed for human red cells (13). Two conclusions can be drawn from this study. First, DNFB produces its effect by interacting directly with the membrane. This is arrived at from the absence of any time delay in the kinetics of onset of inhibition. If DNFB produces its effect by other means, such as interacting with the cell interior, one would expect a time lag in its effect. Second, the

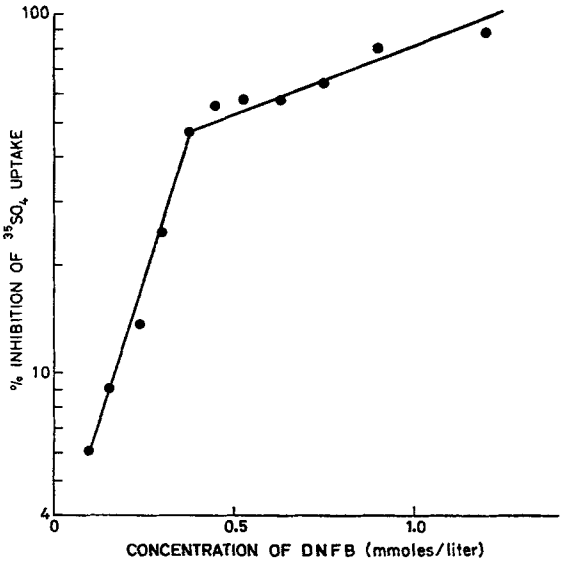

FIGURE 3. Inhibition of $^{35}SO_4$ inward movement by varying concentrations of DNFB in cat red cells. The uptake of $^{35}SO_4$ in incubation medium containing no DNFB and 2.5% methanol was taken as 100%. Methanol alone at this concentration produced 8% inhibition in $^{35}SO_4$ uptake.

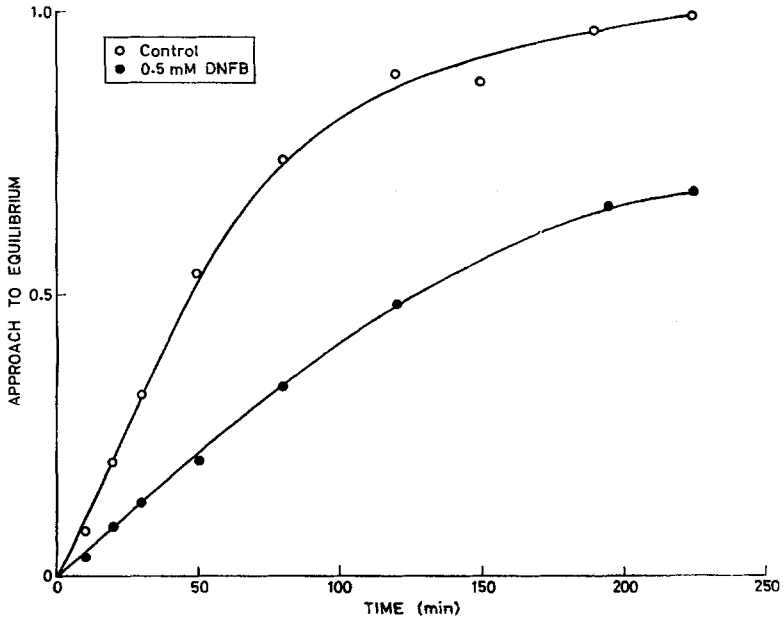

FIGURE 4. The kinetics of inhibition of $^{35}SO_4$ uptake by 0.5 mM DNFB.

effective charge density which regulates anion passage in cat red cells is smaller than the corresponding one for human red cells. In preliminary experiments, DNFB at a concentration of 1 mmole/liter had no effect on the uptake of erythritol-^{14}C, and increased Na influx. This confirms the conclusion of Berg et al. that the primary effect of DNFB on ionic movement is by abolishing the positive charges lining the pores and not on pore size (18).

Effect of Monovalent Anions on $^{35}SO_4$ Uptake

As cation transport in cat red cells is known to be affected by the nature of the anion in the incubating medium (6, 7), $^{35}SO_4$ movement in these cells was studied in the presence of various monovalent anions. In these experi-

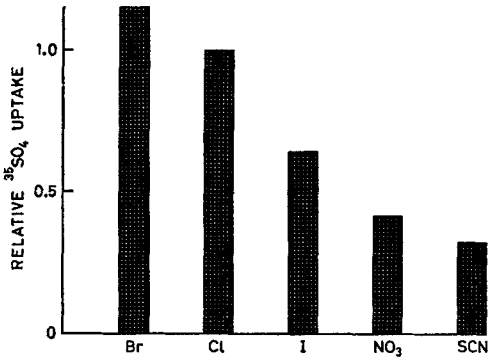

FIGURE 5. The effect of monovalent anions on relative $^{35}SO_4$ uptake in cat red cells

ments, red cells were suspended in a solution identical to the incubation medium except for the replacement of NaCl by the sodium salt of the anion under study. The cells were incubated as usual for about 30 min and then $^{35}SO_4$ was added. A measure of the inward movement of $^{35}SO_4$ was obtained by determining the amount of tracer entering the cells after 90 min. The results of a typical set of experiments are shown in Fig. 5. $^{35}SO_4$ uptake decreases according to the sequence Br > Cl > I > NO_3 > SCN. This sequence is similar to that found for Na uptake in these cells except for the interchange between the positions of Cl and Br (6). $^{35}SO_4$ uptake responds similarly to these anions in both cat and human red cells (10).

To determine the range of effectiveness of the anion thiocyanate in inhibiting $^{35}SO_4$ movement, a series of experiments was carried out in which various degrees of SCN substitution for Cl were employed. Maximal inhibition was achieved when SCN was present at a concentration of 30 mmole/liter.

The kinetics of inhibition of $^{35}SO_4$ inward movement by SCN were also studied. In these experiments, packed red cells were added to medium containing SCN and $^{35}SO_4$ maintained at 38°C. Samples were taken every 10

min for the first 30 min and at longer intervals thereafter. The results of a representative experiment are shown in Fig. 6. As there is no apparent delay in the action of SCN, its effect is produced by direct interaction with the membrane. Another possible explanation for the absence of any time lag is that the presence of SCN alters the membrane potential. This change can take place instantaneously. If this is the case, then one would expect an increase rather than a decrease in $^{35}SO_4$ uptake since the permeability of SCN is surely less than Cl. These anions induce their effects probably by changing

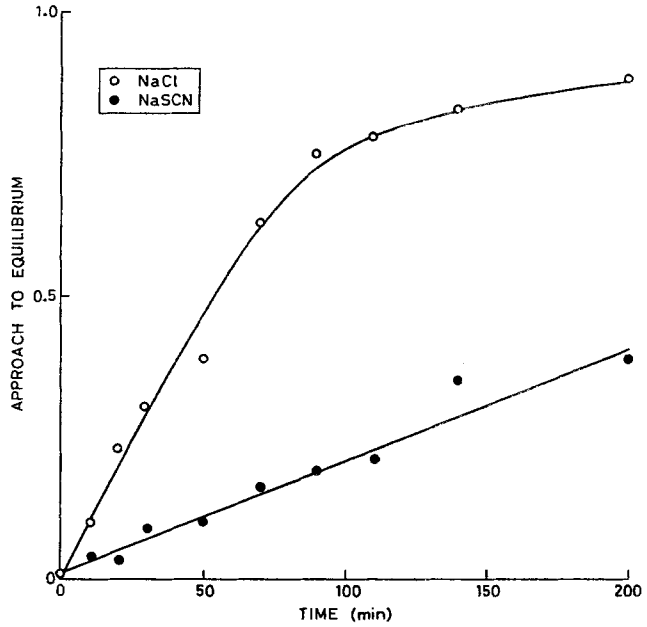

FIGURE 6. The kinetics of inhibition of $^{35}SO_4$ uptake by SCN. NaCl was completely replaced by NaSCN in the incubation medium.

the charge characteristics of the path(s) used by monovalent anions and cations, and thus may cause a shift in their selectivity, making them more selective to K than to Na.

Possible sites of action of these anions are fixed charges on the hydrophilic surfaces of membrane phospholipids. According to Booij (19), such binding can change the packing of carbon chains in the membrane or even induce phase transitions in the lipid structure. As was suggested by Lucy (20), this transition may create pores lined by polar groups. These pores may be more suited for K than for Na movement, explaining acceleration of K influx and diminution of Na influx in cat red cells by these anions. According to this line of reasoning, ion movement must be controlled by the lipid components of the membrane. Changes caused by ion binding, and in particular the crea-

tion of pores, should result in an increase in the permeation of small neutral hydrophilic molecules. This prediction was not borne out experimentally. The uptake of erythritol-^{14}C at different cell volumes was not affected when NaCl was completely replaced by NaSCN in the incubating medium. The results are summarized in Table III. Once again, the dependence of erythritol-^{14}C uptake on cell volume is evident in the table. The apparent 5% increase in erythritol-^{14}C in the presence of NaSCN is probably due to a nonspecific interaction with the membrane. Entry of erythritol-^{14}C into these cells is entirely by passive diffusion due to the absence of a facilitated glucose transport mechanism which can also accommodate erythritol as in human red cells. Furthermore, SCN does not affect the filtration permeability for water in cat red cells (unpublished observation). Therefore, it is unlikely that SCN affects the permeation of small neutral hydrophilic molecules.

TABLE III

EFFECT OF SCN ON ERYTHRITOL-^{14}C UPTAKE AT DIFFERENT CELL VOLUME IN CAT RED CELLS*

Relative cell volume	Relative uptake in		Uptake in NaSCN / uptake in NaCl
	NaCl	NaSCN	
0.94	0.92±0.02	1.01±0.02	1.10±0.01
1.00	1.00±0.03	1.05±0.01	1.05±0.01
1.18	1.20±0.02	1.25±0.01	1.04±0.01
1.30	1.33±0.10	1.31±0.02	0.99±0.05

* Errors represent 1.0 SE of the mean.

Other probable sites of action of these anions are the free amino groups of lysine. Scatchard and Black have shown that the degree of adsorbability of these anions to proton-carrying groups of albumin increases according to the series Cl, Br, NO_3, I, and SCN (21). The order of effectiveness of these anions on $^{35}SO_4$ uptake, with the exception of Br and I, is consistent with this view.

As stated earlier, it is known that Na movement in cat red cells decreases dramatically with increasing cell volume and increases with decreasing cell volume; also, SCN inhibits Na influx and accelerates K influx in these cells. If the action of these anions is as depicted in the preceding analysis, and if volume changes produce some sort of geometrical alteration, then it should be possible, by an appropriate change in cell volume for SCN, to produce the same effect on Na influx (increase) in cat as in human red cells. That this is indeed the case is evident from the results shown in Fig. 7. In these experiments, the cell volume was changed by varying the concentration of NaCl in the control flask and NaSCN in the test flask. The final

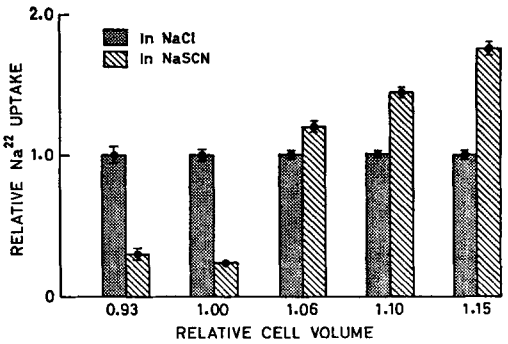

FIGURE 7. Effect of NaSCN on ^{22}Na uptake at different cell volumes in cat red cells.

osmolarity of each suspension was measured to avoid any secondary effect due to difference in cell volume in the control and test flasks. It is clear from the figure that SCN increases ^{22}Na uptake in swollen cat red cells and decreases this uptake in shrunken cells. Moreover, at a relative cell volume of 1.10 or greater, SCN reverses completely the inhibitory effect of cell swelling on ^{22}Na uptake.

This interdependent influence of cell volume and SCN on Na movement, coupled with similar experiments on the selectivity pattern of cat red cell membrane to Li, Na, K, Rb, and Cs under different cell volumes, should give further insight into the molecular basis of selectivity differences between cat and human red cell membranes.

Received for publication 2 June 1971.

BIBLIOGRAPHY

1. GLYNN, I. M. 1957. The ionic permeability of the red cell membrane. *Progr. Biophys.* **8**:242.
2. HOFFMAN, J. F. 1966. The red cell membrane and the transport of sodium and potassium. *Amer. J. Med.* **41**:666.
3. TOSTESON, D. C., and J. F. HOFFMAN. 1961. Regulation of cell volume by active cation transport in high and low potassium sheep red cells. *J. Gen. Physiol.* **44**:169.
4. POST, R. L. 1961. Sodium and potassium transport across the human erythrocyte membrane. *In* Biophysics of Physiological and Pharmacological Actions. A. M. Shanes, editor. Amer. Assn. for the Advancement of Science. Washington, D. C. 19.
5. BERNSTEIN, R. E. 1954. Potassium and sodium balance in mammalian red cells. *Science (Washington).* **120**:459.
6. SHA'AFI, R. I., and W. R. LIEB. 1967. Cation movements in the high sodium erythrocyte of the cat. *J. Gen. Physiol.* **50**:1751.
7. DAVSON, H. 1940. The influence of the lyotropic series of anions on cation permeability. *Biochem. J.* **34**:917.
8. SHA'AFI, R. I., and J. J. HAJJAR. 1971. Sodium movement in high sodium feline red cells. *J. Gen. Physiol.* **57**:684.
9. WIETH, J. O. 1970. Paradoxical temperature dependence of sodium and potassium fluxes in human red cells. *J. Physiol. (London).* **207**:563.

10. WIETH, J. O. 1970. Effect of some monovalent anions on chloride and sulfate permeability of human red cells. *J. Physiol. (London).* **207**:581.
11. SOLOMAN, A. K. 1960. Compartmental methods of kinetic analysis. *In* Mineral Metabolism. C. L. Comar and F. Bronner, editors. Academic Press, Inc., New York. 119.
12. TOSTESON, D. C. 1959. Halide transport in red blood cells. *Acta Physiol. Scand.* **46**:19.
13. PASSOW, H. 1969. Passive ion permeability of the erythrocyte membrane. *Progr. Biophys.* **19**(2):425.
14. ROGERS, T. A. 1961. The exchange of radioactive magnesium in erythrocytes of several species. *J. Cell. Comp. Physiol.* **57**:119.
15. RICH, G. T., R. I. SHA'AFI, T. C. BARTON, and A. K. SOLOMON. 1967. Permeability studies on red cell membranes of dog, cat, and beef. *J. Gen. Physiol.* **50**:2391.
16. PONDER, E. 1948. Hemolysis and Related Phenomena. Grune and Stratton Inc., New York. 84.
17. RICH, G. T., R. I. SHA'AFI, A. ROMUALDEZ, and A. K. SOLOMON. 1968. Effect of osmolality on the hydraulic permeability coefficient of red cells. *J. Gen. Physiol.* **52**:941.
18. BERG, H. C., J. M. DIAMOND, and P. S. MARFEY. 1965. Erythrocyte membrane. Chemical modification. *Science (Washington).* **150**:64.
19. BOOIJ, H. L. 1966. Thoughts about the mechanism of membrane movements. *In* Intracellular Transport. K. B. Warren, editor. Academic Press, Inc., New York. 301.
20. LUCY, J. A. 1968. Ultrastructure of membranes: micellar organization. *Brit. Med. Bull.* **24**:127.
21. SCATCHARD, G., and E. S. BLACK. 1949. The effects of salts on the isoionic and isoelectric points of proteins. *J. Phys. Colloid. Chem.* **53**:88.