29. J. S. Rhim, W. Vass, H. Y. Cho, R. J. Huebner, *Int. J. Cancer* 7, 65 (1971).
30. P. J. Price, A. E. Freeman, W. T. Lane, R. J. Huebner, *Nature (London)* **230**, 144 (1971).
31. J. S. Rhim, D. K. Park, P. Arnstein, R. J. Huebner, E. K. Weisburger, *ibid.* **256**, 751 (1975).
32. C. S. Cooper *et al.*, *ibid.* **311**, 29 (1984).
33. G. Cooper, *Science* **217**, 801 (1982).
34. R. A. Weinberg, *Adv. Cancer Res.* **36**, 149 (1982).
35. M. E. Furth, L. J. Davis, B. Fleurdelys, E. M. Scolnick, *J. Virol.* **43**, 294 (1982).
36. We thank W. Peterson of Children's Hospital of Michigan (Detroit) for performing the karyological analyses of the human epithelial cell lines. We also thank W. Jones of this laboratory for his excellent technical assistance.

26 September 1985; accepted 24 January 1986

Regulation of Erythrocyte Cation and Water Content in Sickle Cell Anemia

CARLO BRUGNARA, H. FRANKLIN BUNN, DANIEL C. TOSTESON

The pathophysiological events in sickle cell disease are critically dependent on the intracellular concentration of hemoglobin S, which varies inversely with cell cation and water content. Erythrocytes of SS homozygotes exposed to oxygen or carbon monoxide decrease their potassium and water content through a pathway for potassium transport that is activated by both cell swelling and decrease in internal *p*H. This pathway is not inhibited by ouabain either with or without bumetanide. When SS erythrocytes were separated according to density, the *p*H- and volume-dependent potassium transport was greatest in the least dense fraction and was reduced in the densest cells. This pathway, which does not depend on polymerization of sickle hemoglobin, may be important in regulating the cation and water content of SS erythrocytes.

THE POLYMERIZATION OF DEOXYHEMOGLOBIN S is the proximate cause of the vaso-occlusive and hematologic manifestations of sickle cell disease. This concerted process is markedly dependent on the intracellular concentration of hemoglobin S (*1*). Therefore, alterations in cell volume would be expected to influence the pathogenesis of sickle cell disease. We recently reported that red cells of patients homozygous for hemoglobin C (CC) can regulate their volume through a pathway for K transport that is not active in normal AA erythrocytes (*2*). This pathway depends on cell volume and *p*H and is not inhibited by ouabain or bumetanide (inhibitors of the Na-K pump and of the Na-K-Cl cotransport). This pathway is probably responsible for the reduced K and water content observed in CC erythrocytes. We have tentatively attributed the activation of the volume-dependent K transport in CC erythrocytes, which does not occur in AA erythrocytes, to the presence of the more positively charged hemoglobin C, perhaps through an electrostatic interaction (*3*) between hemoglobin C and components of the red cell membrane.

Since hemoglobin S, like hemoglobin C, is less negatively charged than hemoglobin A [replacement of the glutamic acid at position 6 in the β chain of hemoglobin A by valine in hemoglobin S and lysine in hemoglobin C (*4*)] and since erythrocyte water content and hemoglobin concentration (MCHC) are important determinants in the pathogenesis of sickle cell disease, we investigated whether erythrocytes containing hemoglobin S also express a volume- and *p*H-dependent K transport pathway.

Experiments were carried out in oxygenated or carbon monoxide–treated erythrocytes from four patients homozygous for sickle cell anemia (SS). To overcome the problems related to heterogeneity of cell cation and water content (*4, 5*), we used either the nystatin technique, to make cells homogeneous for cation and water content, or the Stractan method, to separate red cells according to density (*6*). In brief, the red cell membrane is made permeable to Na and K by exposure to nystatin; the Na, K, and water content are adjusted to desired values; and the nystatin is removed to restore Na and K permeabilities to normal.

When SS red cells were treated with nystatin to make their Na, K, and water contents similar to those of normal AA cells, the ouabain- and bumetanide-resistant K efflux showed a *p*H and volume dependence similar to those described for CC cells. The *p*H-dependence of K efflux gave a bell-shaped curve, with a *p*H optimum of 6.7 to 7.0 and inhibition at both acid and alkaline *p*H. Cell swelling induced by hyposmotic media (220 instead of 300 mos*M*) resulted in a four- to fivefold increase in K efflux.

Similar studies were performed in SS cells isolated with the Stractan density gradients. Three fractions are obtained: the top fraction contains the lightest cells, including reticulocytes; the middle fraction contains the mature discoid cells; and the bottom fraction contains the most dense cells, including irreversibly sickled cells (*6*).

The ouabain- and bumetanide-resistant K efflux was different in the three fractions of SS erythrocytes and was differently affected by changes in cell volume (obtained by altering the osmolarity of the media) (Table 1). In the top fraction, the K efflux was stimulated by hyposmolarity and reduced to almost normal values by hypertonicity. In contrast, the K efflux from the bottom fraction was small and showed much less volume dependence. However, the different hemoglobin concentrations and cation and water content of the three fractions could have been responsible for these differences. Therefore the cells were first separated with

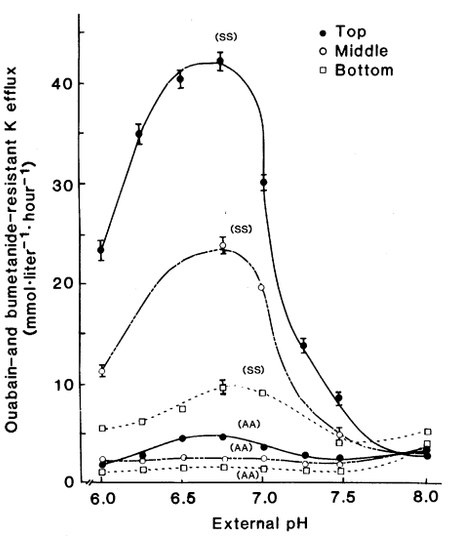

Fig. 1. Dependence on *p*H of the ouabain- and bumetanide-resistant K efflux from the top, middle, and bottom fractions of SS and AA erythrocytes separated on a discontinuous Stractan density gradient. The red cells had been incubated earlier with carbon monoxide. After separations, the fractions were treated with nystatin to obtain similar Na, K, and water contents. MCHC values before and after nystatin treatment for top, middle, and bottom fractions of SS cells were 32.6 and 31.1 g/dl, 37.8 and 32.1 g/dl, and 48.6 and 35.3 g/dl, respectively, and for the three fractions of AA cells were 31 and 31 g/dl, 32.6 and 32.2 g/dl, and 34.1 and 32.2 g/dl, respectively. K efflux was measured in room air with media containing 140 m*M* NaCl, 1 m*M* $MgCl_2$, 10 m*M* tris-MOPS (*p*H 6.0 to 8.0), 10 m*M* glucose, 0.1 m*M* ouabain, and 0.01 m*M* bumetanide. The incubation times were 5 and 25 minutes at 1% hematocrit (*6*). Results are means ± SEM of the fluxes. Similar results were obtained in two other patients and in one normal control.

C. Brugnara and D. C. Tosteson, Department of Physiology and Biophysics, Harvard Medical School, Boston, MA 02115.
H. F. Bunn, Laboratory of the Howard Hughes Medical Institute, Hematology Division, Brigham and Women's Hospital, Harvard Medical School, Boston, MA 02115.

Table 1. Ouabain- and bumetanide-resistant K efflux from SS erythrocytes separated according to density. Stractan density gradients were used according to the method developed by Clark *et al.* (*6*). The K efflux was measured at three different osmolarities in media containing 100 m*M* NaCl, 1 m*M* $MgCl_2$, 10 m*M* tris-MOPS (*p*H 7.4 at 37°C), 10 m*M* glucose, 0.1 m*M* ouabain, and 0.01 m*M* bumetanide. The osmolarity of the medium (220 mos*M*) was increased to 300 mos*M* and 400 mos*M* with choline chloride (50 m*M* and 100 m*M*, respectively). The incubation times were 5 and 25 minutes at 1% hematocrit. Details of the method are given in (*2*). Results are means ± SD for three patients. Erythrocyte cation content is measured as millimoles per liter of cells.

Fraction	MCHC (g/dl)	Erythrocyte cation content (mmol · liter^{-1})			Ouabain- and bumetanide-resistant K efflux (mmol · liter^{-1} · hour^{-1})		
		Na	K	Total	220 mos*M*	300 mos*M*	400 mos*M*
Top	32.4 ± 0.5	12.1 ± 5.5	105.4 ± 5.0	117.5 ± 1.0	30.5 ± 9.0	4.2 ± 0.8	2.1 ± 0.3
Middle	38.3 ± 1.5	29.5 ± 16.0	70.0 ± 17.6	99.4 ± 1.6	4.2 ± 0.5	2.3 ± 1.0	1.7 ± 0.4
Bottom	45.6 ± 2.3	38.4 ± 10.5	35.6 ± 6.0	73.6 ± 4.1	1.5 ± 0.2	1.3 ± 0.5	1.1 ± 0.4

the Stractan density gradients and then the cation and water content were changed by the nystatin method (*2*). This procedure yielded cells with similar cation and water content and similar hemoglobin concentrations in the three different fractions. The *p*H and volume dependence of the K efflux were then studied. The *p*H-dependent K efflux was greater in the top fraction (Fig. 1). All the fractions had K efflux values greater than those of the corresponding fractions of the AA control cells. However, in the least dense fraction of AA control cells, the K efflux had a *p*H dependence qualitatively similar to that of SS cells (Fig. 1). Similar results were obtained for the volume dependence of K efflux.

To show that SS erythrocytes can effectively reduce their cation and water content through this pathway, we performed experiments with the top fraction of SS erythrocytes or the whole population of SS red cells. In both cases, the K and water content of the cells were increased by the nystatin technique. The cells were then incubated at 37°C in room air in a medium with Na and K concentrations similar to those of plasma. At different incubation times (0 to 6 hours) a portion of cells was washed, and determinations of cell Na and K content, MCHC, and phthalate density distribution (*7*) were carried out. SS cells incubated under these conditions shrank toward the original volume (Fig. 2). This response was not blocked by ouabain or bumetanide and was explained primarily by K loss.

These experiments indicate that, like the CC cells, swollen SS erythrocytes shrink through a ouabain- and bumetanide-resistant K and water loss. The expression of this K transport pathway in CC and SS cells suggests that less negatively charged hemoglobins participate in the activation of this pathway. Further research is necessary to clarify whether this property is a common feature of all cells containing hemoglobin less negatively charged than hemoglobin A, or whether the system is activated only by hemoglobins with a substitution at position 6 of the β chain of globin.

The correlation between the magnitude of the volume- and *p*H-dependent K transport pathway and cell density raises another possibility. In both SS and AA cells, the system is expressed maximally in the least dense cells and minimally in the most dense cells. It will be important to investigate whether this K transport pathway is expressed in all cells with a low density and how its expression relates to cell age. At present, we cannot reject the hypothesis that, in addition to the presence of less negatively charged hemoglobin, cell age is an important factor affecting the expression of this transport system in human red cells.

These experiments show that SS erythrocytes can decrease their water content when the hemoglobin is ligated with O_2 or CO and therefore not sickled. In contrast, most previous work on cation permeability of SS cells has focused on changes induced by deoxygenation and sickling. In 1952, Tosteson *et al.* (*8*) showed that sickling was accompanied by a large increase in the Na and K permeabilities. When SS cells were depleted of adenosine triphosphate (ATP), the K loss was larger than the Na gain, and the water content decreased (*9*). Internal *p*H also plays an important role in sickling. First, acid shifts in internal *p*H have been shown to induce more sickling than could be expected from the change in the oxygen affinity due to the Bohr effect (*10*); and second, the deoxygenation-induced K efflux has also been shown to be dependent on *p*H (*11*). The high Ca content of sickle cells has led many investigators to propose a role for the Ca-activated K channel in reducing the cell K and water content (*9, 12*). However, there is recent evidence that most of the Ca in SS cells is stored in cytoplasmic inside-out

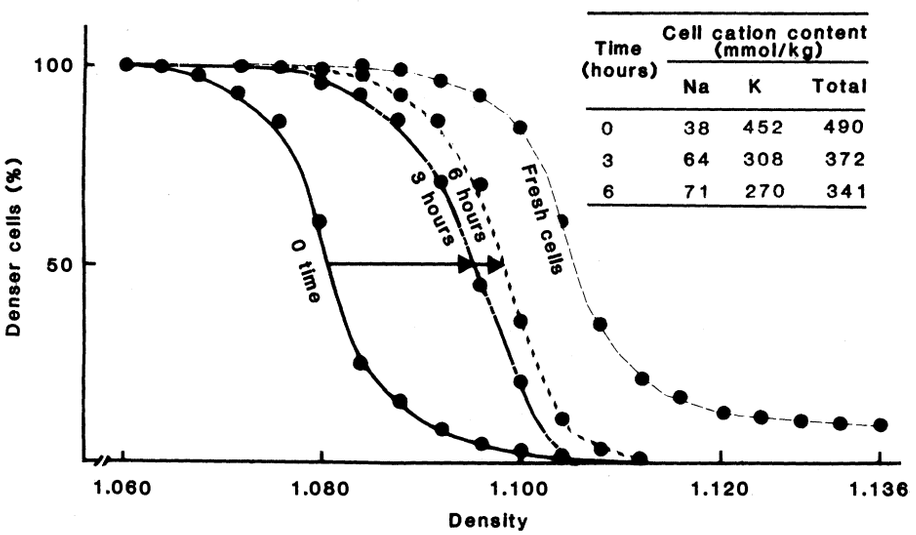

Fig. 2. Erythrocyte density profile of swollen SS cells incubated in isotonic medium. The nystatin technique was used to make SS erythrocytes swollen compared to their normal volume. The cells were then incubated at 37°C in room air, in a medium containing 140 m*M* NaCl, 4 m*M* KCl, 1 m*M* $MgCl_2$, 1 m*M* phosphate, 10 m*M* tris-MOPS (*p*H 7.4) at 37°C, 10 m*M* glucose, 0.1 m*M* ouabain, and 0.01 m*M* bumetanide. At given times the cells were washed with choline washing solution, and determinations of cell Na, K, MCHC, and phthalate density distribution were carried out. Twenty different phthalate densities (range 1.060 to 1.136) were used. The density profile of the same SS cells before nystatin treatment is included for comparison. Inset: cell cation content, in millimoles per kilogram of hemoglobin, for cells incubated in the presence of ouabain and bumetanide. Similar results were obtained when ouabain and bumetanide were omitted from the incubation medium: cell cation content at 3 hours was Na, 32; K, 343; and total, 375 mmol/kg; and at 6 hours was Na, 32; K, 305; and total, 337 mmol/kg. Similar results were obained for the top fraction of SS erythrocyte isolated with Stractan.

vesicles and therefore unable to activate membrane transport of K (*13*). Moreover, the increase in Na and K permeabilities induced by short periods of deoxygenation has no effect on the water content and hemoglobin S concentration (*14*), and it is probably produced by membrane damage induced by hemoglobin S polymerization, rather than by activation of specific transport pathways.

Earlier studies have not systematically addressed the problem of regulation of cell volume and cation transport in oxygenated SS cells. In one report (*6*), the oxygenated, low-density, reticulocyte-rich fraction of SS cells was found to have a large ouabain-resistant K efflux. We have now shown that this increased K transport takes place through a *p*H- and volume-dependent system, maximally active in the least dense fraction but present also in other fractions of SS cells. When SS erythrocytes are swollen at *p*H 7.4 or incubated in acid medium, they can effectively decrease their water content and increase their MCHC. Because cellular dehydration makes a major contribution to the abnormal rheology of SS cells (*15*), it will be important to identify the role of this K movement in the formation of irreversibly sickled cells. The effect of deoxygenation on the *p*H- and volume-dependent K transport pathway and the relation to the increased K and Na permeabilities induced by sickling await further investigation. Our findings suggest that in SS erythrocytes a reduction in cell volume (with a parallel reduction in cell deformability) can be accomplished not only by repetitive cycles of polymerization and depolymerization, but also by a decrease in internal *p*H when hemoglobin is in the fully ligated state.

REFERENCES AND NOTES

1. W. A. Eaton, J. Hofrichter, P. D. Ross, *Blood* **47**, 621 (1976); F. A. Ferrone, J. Hofrichter, W. A. Eaton, *J. Mol. Biol.* **183**, 591 and 611 (1985).
2. C. Brugnara, A. Kopin, H. F. Bunn, D. C. Tosteson, *J. Clin. Invest.* **75**, 1608 (1985).
3. R. K. Kaul and H. Köhler, *Klin. Wochenschr.* **61**, 831 (1983).
4. H. F. Bunn and B. G. Forget, *Hemoglobin: Molecular, Genetic and Clinical Aspects* (Saunders, Philadelphia, 1986).
5. M. E. Fabry and R. L. Nagel, *Blood Cells* **8**, 9 (1982); D. K. Kaul, M. E. Fabry, P. Windisch, S. Baez, R. L. Nagel, *J. Clin. Invest.* **72**, 22 (1983).
6. M. R. Clark, C. E. Morrison, S. B. Shohet, *J. Clin. Invest.* **62**, 329 (1978).
7. G. P. Rodgers, A. N. Schechter, C. T. Noguchi, *J. Lab. Clin. Med.* **106**, 30 (1985).
8. D. C. Tosteson, E. Shea, R. C. Darling, *J. Clin. Invest.* **31**, 406 (1952); D. C. Tosteson, E. Carlsen, E. T. Dunham, *J. Gen. Physiol.* **39**, 31 (1955).
9. B. E. Glader and D. G. Nathan, *Blood* **51**, 983 (1978).
10. R. M. Bookchin, T. Balazs, L. C. Landau, *J. Lab. Clin. Med.* **87**, 597 (1976).
11. E. F. Roth, R. L. Nagel, R. M. Bookchin, *Am. J. Hematol.* **11**, 19 (1981).
12. L. R. Berkowitz and E. P. Orringer, *J. Clin. Invest.* **68**, 1215 (1981); *Blood Cells* **8**, 283 (1982).
13. V. L. Lew *et al.*, *Nature (London)* **315**, 586 (1985).
14. L. R. Berkowitz and E. P. Orringer, *Am. J. Physiol.* **249**, C208 (1985).
15. M. R. Clark, N. Mohandas, S. B. Shohet, *J. Clin. Invest.* **65**, 189 (1980); N. Mohandas, W. M. Phillips, M. Bessis, *Semin Hematol.* **16**, 95 (1980).

16. Supported by grants from National Institutes of Health (HL-34671 and HL-16927) and by the Howard Hughes Medical Institute.

18 September 1985; accepted 24 January 1986

Suprachiasmatic Nucleus Vasopressin Messenger RNA: Circadian Variation in Normal and Brattleboro Rats

GEORGE R. UHL AND STEVEN M. REPPERT

In situ hybridization of an oligonucleotide probe complementary to vasopressin messenger RNA (mRNA) in sections from normal or Brattleboro rat hypothalami revealed hybridization densities in each of three vasopressin-rich nuclei: the supraoptic, paraventricular, and suprachiasmatic. When entrained to a daily light-dark cycle, each rat strain displayed diurnal variation in hybridizable mRNA in the suprachiasmatic, but not in the supraoptic or paraventricular nuclei. The higher values for suprachiasmatic mRNA in the morning correlate well with previously elucidated morning increases in vasopressin immunoreactivity in the cerebrospinal fluid. These results (i) support the utility of in situ hybridization techniques for elucidating physiological influences on regional peptidergic function, (ii) are consistent with a prominent role for vasopressinergic suprachiasmatic neurons in generating the cerebrospinal fluid vasopressin rhythm, and (iii) suggest that regulation of this mRNA rhythm is not dependent on release of intact peptide.

CONCENTRATIONS OF THE NEUROpeptide vasopressin in the cerebrospinal fluid (CSF) vary in a circadian rhythm, with morning levels three to ten times those at night (*1, 2*). The persistence of this rhythm in blinded animals indicates that it is generated by an endogenous circadian pacemaker; it is also independent of the osmotic regulation of plasma vasopressin (*3*). Vasopressin-containing neurons in three hypothalamic zones—the paraventricular (PVN), supraoptic (SON), and suprachiasmatic nuclei (SCN)—are the major candidates for the generation of this CSF vasopressin rhythmicity (*4*).

In situ hybridization methods can allow assessment of peptide messenger RNA (mRNA) densities in various brain regions (*5*). We used a recently developed method to study diurnal vasopressin (prepropressophysin) mRNA rhythms and to ascertain whether the cellular contents of transmitter-specific mRNA's reflect the functional activity of a particular neuronal population. This appears to be the case for several other peptidergic endocrine systems, in which variations in a cell's secretory activities parallel alterations in the expression of the gene for its secreted product (*6*).

In addition, this method could provide insight into the identity of the cell groups responsible for the generation of CSF vasopressin rhythmicity. Cells in the SCN are heavily implicated in circadian cyclicity by a variety of lesion, metabolic, and electrophysiologic studies (*7*). However, these neurons contain only a modest fraction of total hypothalamic vasopressin (*8*). The suprachiasmatic pacemaker could therefore act either directly by regulating vasopressin release from SCN neurons or indirectly by modulating other vasopressin-containing nuclei receiving SCN input, such as the PVN.

Finally, we could examine Brattleboro rats for regional diurnal vasopressin mRNA cyclicity. These animals display diabetes insipidus, lack hypothalamic vasopressin, and have a deletion mutation in a structural portion of their vasopressin gene (*9*). Several groups have also searched for regulatory defects in the Brattleboro gene, and have found either reduced or normal hypothalamic vasopressin mRNA levels in the Brattleboro animals (*10–12*). Interpretation of these studies is complicated, however, by the difficulty in matching the degrees of dehydration in Brattleboro and control groups. Examination of the diurnal vasopressin rhythm in the SCN can obviate this problem, since vasopressin mRNA in this

G. R. Uhl, Department of Neurology and Howard Hughes Neuroscience Group, Massachusetts General Hospital, and Harvard Medical School, Wellman 405, Massachusetts General Hospital, 50 Blossom Street, Boston, MA 02114.
S. M. Reppert, Children's Service and Laboratory of Developmental Chronobiology, Massachusetts General Hospital, and Harvard Medical School, Boston, MA 02114.