

## Hydration of sickle cells using the sodium ionophore Monensin. A model for therapy.

M R Clark, ... , N Mohandas, S B Shohet

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### Research Article

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# Hydration of Sickle Cells Using the Sodium Ionophore Monensin

## A MODEL FOR THERAPY

MARGARET R. CLARK, NARLA MOHANDAS, and STEPHEN B. SHOHET, with the technical assistance of ROBERT M. HOESCH, *Departments of Laboratory Medicine and Medicine, and Cancer Research Institute, University of California, San Francisco, California 94143*

**ABSTRACT** Mean cell hemoglobin concentration (MCHC) is thought to have an important influence in sickle cell disease, both through the strong dependence of sickling rates on hemoglobin S concentration, and through the profoundly limiting effect of high MCHC on the rheologic competence of oxygenated, irreversibly sickled cells (ISC). Recent studies have tested the ability of antidiuretic hormone to reduce sickle cell MCHC by reducing plasma sodium (Na) and osmolality. An alternative means of reducing MCHC is to elevate intracellular cation content, rather than to depress extracellular cation concentration. In an effort to do this, we have treated sickle cells with Monensin, an antibiotic that selectively enhances membrane Na permeability. At submicromolar concentrations, Monensin substantially reduced the MCHC of whole sickle blood and isolated ISC, causing an improvement in cell deformability. Monensin's effectiveness in producing a controlled increase in erythrocyte water content suggests that agents that selectively increase membrane Na permeability could be therapeutically useful.

## INTRODUCTION

Over the past several years, it has become evident that polymerization of deoxyhemoglobin S within an eryth-

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Address correspondence to Dr. Clark, M-1282 Cancer Research Institute, University of California, San Francisco, CA 94143.

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rocyte can have profoundly deleterious effects on the cell's water content. The drastically reduced water content of irreversibly sickled cells (ISC)<sup>1</sup> (1, 2), presumed to have resulted from prolonged sickling, severely compromises the deformability of these cells, even when they are fully oxygenated (3). In addition, because of the extreme dependence of hemoglobin S polymerization rates on hemoglobin concentration (4), even modest dehydration of discoid sickle cells would greatly increase their propensity to sickle at reduced oxygen tension. Noguchi and Schechter (5) have found that sickle cells contained some residual polymeric hemoglobin even at oxygen saturations >95%. Because mean arterial oxygen saturation values in sickle cell patients are <100%, they propose that a high hemoglobin S concentration leads to persistence of deoxyhemoglobin S polymers even in arterial blood, perhaps greatly shortening or eliminating the "delay time" for hemoglobin S gelation when cells encounter a low oxygen tension.

Because of the critical role of hemoglobin concentration in the sickling process, it has been proposed that a reduction of mean cell hemoglobin concentration (MCHC) of sickle cells below the normal value should provide an antisickling effect (6). On the basis of such considerations, several investigators have initiated clinical studies in which sickle cell patients were treated with antidiuretic hormone and a low sodium (Na) diet, to reduce plasma osmolality and Na concentration, in hopes of producing a secondary reduc-

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<sup>1</sup> *Abbreviations used in this paper:* BSKG, buffered saline containing potassium and glucose; DI, deformability index; DIDS, 4,4'-diisothiocyano-2,2'-stilbenedisulfonic acid; Hct, hematocrit; ISC, irreversibly sickled cells; MCHC, mean cell hemoglobin concentration.

tion in MCHC (7-9). Unfortunately, these trials have had only limited success, partly because of the difficulty in safely maintaining low plasma Na concentrations (8), and partly because this is a relatively ineffective means of increasing erythrocyte water content. We have attempted a somewhat different approach to the problem by inducing a Na-selective leak in the erythrocyte membrane using the antibiotic Monensin (10), a drug in current veterinary use. Because of the large plasma reservoir of Na, this approach should have an inherently greater capacity for sustained cell hydration. In this study, we have found that submicromolar concentrations of Monensin are effective in increasing the Na and water content of ISC and discoid sickle cells. Furthermore, this increased hydration produces a substantial improvement in the whole cell deformability of sickle cell blood samples. These results provide encouragement for the idea that agents that cause a selective increase in Na permeability could provide an additional therapeutic tool in sickle cell disease.

## METHODS

Erythrocytes were treated with Monensin under two separate sets of conditions: in plasma-free buffered solutions and in suspensions containing heated plasma. For treatment in the absence of plasma, erythrocytes were washed three times in buffered saline containing potassium and glucose (BSKG: 134 mM NaCl, 5 mM KCl, 8.6 mM Na<sub>2</sub>HPO<sub>4</sub>, 1.4 mM NaH<sub>2</sub>PO<sub>4</sub>, and 11 mM glucose adjusted to 290-295 mosmol/kg and pH 7.4) and resuspended to ~5% hematocrit (Hct). A 2-mM stock solution of Na-Monensin (Calbiochem-Behring Corp., La Jolla, CA) in ethanol was then added after dilution in BSKG to give the desired final concentration. (This resulted in addition of only  $5 \times 10^{-2}$   $\mu$ l of ethanol to 10 ml of cell suspension for a final Monensin concentration of 10 nM.) For treatment in the presence of plasma, heparinized blood samples were first centrifuged, and the plasma was heated to 56°C for 1 h to inactivate the lecithin-cholesterol acyltransferase enzyme, which causes echinocytosis during prolonged incubation (11). While the plasma was being heated, the erythrocytes were washed in BSKG to remove residual plasma and the buffy coat. The washed cells were then recombined with a mixture of heated plasma and BSKG. A few experiments were performed in which cells were suspended in plasma alone. In these experiments, 10 mM glucose was added, and the cells were incubated under an atmosphere of 5% CO<sub>2</sub> in air to maintain the pH.

The effect of Monensin on cellular ion and water content was monitored by measuring MCHC, intracellular Na and K content, and by analysis of cell density distribution on Stractan density gradients. MCHC was determined from spectrophotometric measurements of hemoglobin as the cyanomethemoglobin complex and from spun Hcts. Intracellular Na and K content were determined by flame photometric analysis of aliquoted erythrocytes washed in ice-cold, isotonic Tris-buffered magnesium chloride solution (10 mM Tris-HCl, pH 7.4).

Density analysis was performed by centrifuging the cells

on discontinuous gradients of Stractan II (St. Regis Paper Co., New York, NY) (12). The gradients covered a density range from 1.065 to 1.139 g/ml, in increments of ~0.009 g/ml. Quantitative evaluations of the cell density distribution were obtained by removing successive layers from the gradient with a Pasteur pipet and measuring their hemoglobin content.

Whole cell deformability was measured using the ektacytometer, a laser-diffraction couette viscometer (13). This instrument provides a deformability index (DI) that is derived from photometric analysis of the laser diffraction image generated by the erythrocytes in the viscometer shear field. The DI value is proportional to the average ellipticity of a uniform population of deforming cells (13) and also provides a measure of the proportion of nondeforming cells in a mixture of deformable and nondeformable cells. We have previously shown that by measuring cell deformability in isotonic and hypotonic media, it is possible to determine whether cells are initially dehydrated or have a reduced surface area-to-volume ratio (13). Dehydrated cells deform poorly in isotonic medium because of their elevated hemoglobin concentration and high intracellular viscosity. Their deformability is improved in hypotonic medium, in which they take up water and reduce their MCHC. In contrast, cells whose surface area-to-volume ratio is reduced (via either membrane loss or volume increase) show little or no impairment of their isotonic deformability, but show an abnormal reduction in hypotonic deformability. In the present studies, deformability was measured at 290 and 210 mosmol/kg to determine the hydration status of the samples.

Monensin has been shown to transport Na across the membrane by exchanging Na ions for protons (10). Thus, to determine whether Monensin treatment had significantly disturbed the intracellular pH, we measured the pH of packed, freeze-thawed, Monensin-treated cells. In addition, because exchange of chloride for hydroxyl ions through the anion channel allows the erythrocyte to dissipate a pH gradient across the membrane (14), we performed experiments using the anion channel blocking agent, 4,4'-diisothiocyano-2,2'-stilbenedisulfonic acid (DIDS) (15), to provide further understanding of Monensin's effects.

## RESULTS

Treatment of sickle erythrocytes with surprisingly low concentrations of Monensin resulted in a dose-dependent increase in Na content (Fig. 1 A). A simultaneous decrease in intracellular K was also seen, but it was much smaller than the increase in Na, leading to a net increase in total cation content. The increase in total cation content entailed an increase in cell water, reflected in a Monensin dose-dependent reduction in MCHC (Fig. 1 B). For sickle cells, this Monensin-induced increase in cell ion and water content was accompanied by an increase in average cell deformability (Fig. 1 B). These experiments showed that for washed sickle cells, Monensin concentrations in the range of 10 to 20 nM gave the maximum improvement in cell deformability. Incubation for 23 h in 20 nM Monensin resulted in 17% hemolysis, compared to 7% hemolysis in the control sample without Monensin.

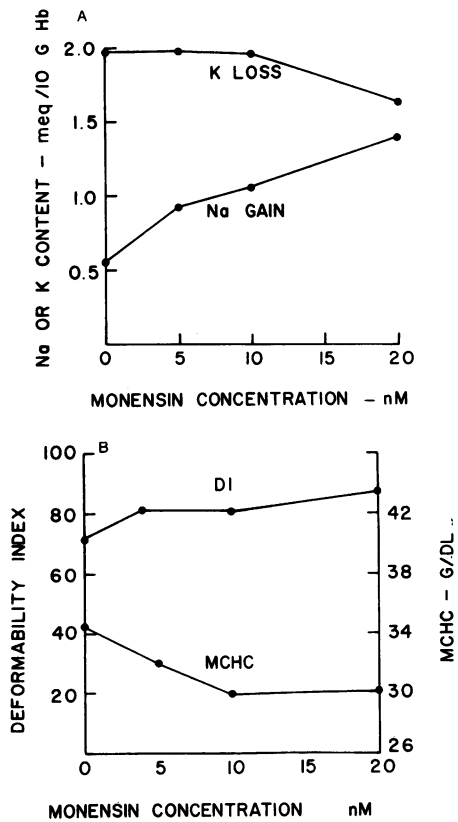


FIGURE 1 A. Changes in Na and K content of washed SS erythrocytes as a function of Monensin concentration. Cells were incubated for 6 h at 37°C in BSKG containing the indicated concentrations of Monensin. At 20 nM concentration, the increase in Na content was 2.7 times the decrease in K content. B. Corresponding changes in MCHC and whole cell deformability at 125 dyn/cm<sup>2</sup> shear stress as a function of Monensin concentration. Increase of total Na plus K content and osmotically driven water caused a Monensin dose-dependent reduction in MCHC. The resultant decrease in intracellular viscosity enabled the initially poorly deformable sickle cells to deform more readily, as indicated by an increase in the ektacytometric DI. DI is given as the percentage of the instrument reading for normal cells at the same shear stress.

Cells incubated with 10 nM Monensin showed 8% hemolysis under these conditions. Above 20 nM concentration, Monensin caused excessive water uptake, with eventual complete hemolysis.

Monensin also increased the Na content of normal cells, while causing minimal changes in K content (Table I a). The ability of Monensin-treated normal cells to deform in isotonic medium was unaffected for incubation periods up to 6 h. However, Monensin treatment did cause a time- and concentration-dependent reduction in hypotonic deformability (Table I b). This change was consistent with moderate cell swell-

ing, resulting in a reduced surface area-to-volume ratio.

To provide additional understanding of Monensin's effect on cell water content, we subjected Monensin-treated cells to density analysis on Stractan gradients. Fig. 2 shows the density distributions of samples of cells from a patient who had homozygous sickle cell disease (SS), after a 4-h incubation with and without Monensin. The histograms show the distributions for cells treated with 0, 5, 10, and 20 nM Monensin. The solid line encloses the distribution for the 20-nM sample, and the dashed line encloses the distribution for the untreated sample. A progressive shift of the entire distribution pattern to lower density occurred with increasing Monensin concentration. Thus, at 20 nM Monensin, there was a large increase in low density cells and a substantial reduction in very high density cells.

When sickle or normal cells were treated with 20 nM Monensin for 7 h, the pH of freeze-thawed, packed cells was the same as for untreated cells. When the anion channel was blocked with DIDS, however, Monensin caused an increase in intracellular pH from 6.89 to 7.10 for sickle cells and from 7.23 to 7.45 for normal cells. Moreover, the inclusion of DIDS also limited the

TABLE I  
Effect of Monensin on Ion Content and Deformability of Normal Cells

Incubation period	Monensin concentration	(a) Erythrocyte cation content		(b) Erythrocyte deformability		
		Na	K	DI <sub>290</sub>	DI <sub>210</sub>	
h	nM	meq/10 g Hb		%		
0	0	0.17	2.81	100	78	
	2	0	0.17	2.76	100	83
		5	0.55	2.81	101	77
		10	0.76	2.82	101	73
		20	1.05	2.75	100	64
4	0	0.18	2.82	100	89	
	5	0.66	2.71	102	77	
	10	0.88	2.58	101	66	
	20	1.24	2.56	101	53	
		6	0	0.18	2.76	101
6	5	0.76	2.83	99	72	
	10	1.08	2.70	101	56	
	20	1.47	2.51	99	41	

Washed normal cells were incubated at 37°C in BSKG with and without Monensin at various concentrations for the indicated periods. Cell deformability was measured at 290 and 210 mosmol/kg and is expressed as the percentage of the DI of the untreated cells before incubation.

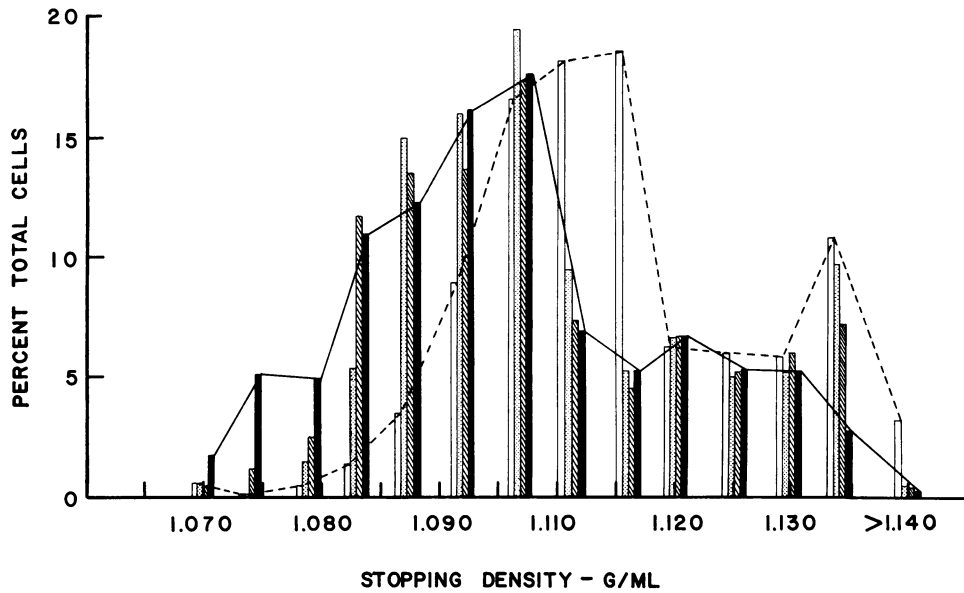


FIGURE 2 Stractan gradient density distributions for SS cells treated with Monensin. The heights of the bars show the percentage of total sample hemoglobin trapped in successive gradient interfaces. The position of the bars along the x-axis corresponds to the density of the layer above which the cells rested. Successive shifts of the population density to lower values were seen for Monensin concentrations of 5 nM (dotted bars), 10 nM (hatched bars), and 20 nM (black bars), as compared with untreated, incubated control cells (open bars). To facilitate comparison of the overall density distribution, lines have been drawn to connect the tops of the bars for control (dashed line) and 20-nM (solid line) samples.

Monensin-mediated uptake of Na and increase in total cation content. DIDS/Monensin-treated normal cells increased their total cation content from 3.22 to only 3.33 meq/10 g Hb, whereas cells treated with Monensin alone contained 3.63 meq/10 g Hb after 7 h. Consistent with this difference in cation content, DIDS also prevented the swelling ordinarily associated with Monensin treatment.

All of these initial experiments had been performed with washed cells, but it was important to determine whether Monensin would be effective in the presence of plasma. It was found that suspending sickle cells in plasma, or in solutions containing a high proportion of plasma, increased ~10-fold the Monensin concentration required to cause appreciable hydration. Fig. 3 shows a representative time course for changes in sickle cell deformability during such treatment. Monensin at 100 nM concentration caused a gradual improvement in cell deformability which was maintained for 24 h. Elevation of the Monensin concentration to 250 nM gave an initial improvement in deformability, but by the end of the 24-h incubation, cell deformability was lower than before Monensin treatment, an effect that was shown by hypotonic deformability measurements to be the result of excessive water up-

take. Monensin at 50 nM concentration had no appreciable effect on cell deformability (data not shown).

The reduction in Monensin's effectiveness in the presence of plasma suggested that the drug could bind to plasma constituents and therefore was likely to distribute between plasma and erythrocytes. Thus, it was of interest to know how long the effects of Monensin

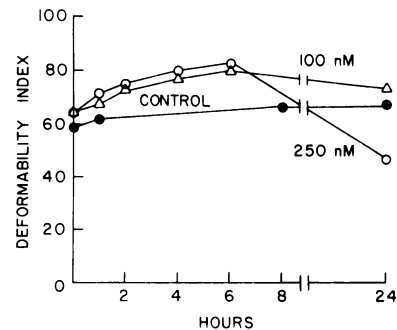


FIGURE 3 Time course of changes in the DI at 125 dyn/cm<sup>2</sup> shear stress for SS cells treated with Monensin in the presence of plasma. Cells were incubated for the indicated periods in a 2:1 mixture of BSKG:heated plasma containing 0, 100, and 250 nM Monensin. Monensin at 50 nM concentration had no effect on DI.

TABLE II  
Effect of Incubation with Plasma on Normal Cells Previously Hydrated with Monensin

	Na	K	DI <sub>200</sub>	DI <sub>210</sub>
	meq/10 g Hb			
Before Monensin treatment	0.25	2.89	100	78
After incubation with 10 nM Monensin for 4 h	0.84	2.74	96	66
After resuspension in BSKG/plasma				
4 h	0.65	2.86	100	71
8 h	0.48	2.84	100	68

Washed normal cells were incubated at 37°C for 4 h at 10% Het in BSKG containing 10 nM Monensin. The BSKG was then replaced with a mixture of 2 parts buffer containing glucose (BSKG): 1 part autologous plasma without Monensin, and the incubation was continued for another 8 h.

might persist after Monensin-treated cells were resuspended in plasma. To obtain information on this question, we incubated normal cells in the presence of 10 nM Monensin for 4 h, then replaced the buffer containing Monensin with a mixture of buffer and plasma and continued the incubation. Measurement of intracellular cations and cell deformability (Table II) showed that increased cell Na and water content persisted for up to 8 h after removal of the drug.

Density profiles of Monensin-treated cells had suggested that the entire sickle cell population responded more or less uniformly to Monensin. To test this pos-

sibility more directly, we isolated subpopulations of discoid and ISC-rich sickle cells and treated them separately with the drug. As summarized in Table III, both types of cells gained substantial amounts of Na while undergoing only slight changes in K content. Both showed substantial reductions in MCHC as a result of Na and water influx. Because of this reduction in MCHC, the ISC-rich cell populations improved their deformability. As previously observed with normal cells, the discoid cells showed virtually no change in isotonic deformability, implying that the degree of swelling they had undergone had not compromised their ability to deform in isotonic medium. The hypotonic deformability of both discoid cells and ISC was reduced at 50 nM Monensin after a 12-h incubation.

An interesting difference was noted in the response to Monensin of cells doubly heterozygous for hemoglobins S and C as compared to homozygous SS cells. As illustrated by the deformability curves in Fig. 4, the initially depressed deformability curve of the SC cells became virtually identical to the curve for normal control cells after Monensin treatment. In contrast, deformability curves of homozygous SS cells whose initial deformability index (DI) was <80% of control were not completely transformed to the normal level at any Monensin concentration, although some improvement was always seen (the average increase in DI for seven such experiments was 24% of the initial value.) This difference in response to Monensin was mimicked by the response of SC and SS cell deformability to a reduction in suspending medium osmolal-

TABLE III  
Effect of Monensin on Separated ISC and Discoid Sickle Cell Populations

	Concentration of Monensin	MCHC	DI <sub>200</sub>	DI <sub>210</sub>	Na	K
	nM	g/dl	%	%	meq/10 g Hb	
Discoid cells						
Pre-incubation	—	35.4	100	98	0.40	2.66
12 h	0	31.5	100	99	0.33	2.97
12 h	10	29.8	100	95	0.60	2.76
12 h	50	29.3	98.1	78	1.38	2.34
ISC-rich cells						
Pre-incubation	—	40.3	56.3	76	0.62	1.58
12 h	0	37.0	64.9	73	0.54	1.84
12 h	10	35.8	68.6	77	0.76	1.74
12 h	50	30.2	78.1	67	1.62	1.76

Blood from a homozygous SS patient was separated on Stractan gradients into subpopulations of mature discoid and dense ISC-rich cells. The washed cells were then incubated in a mixture of 1 part heated plasma: 2 parts BSKG, with or without Monensin at the indicated concentrations.

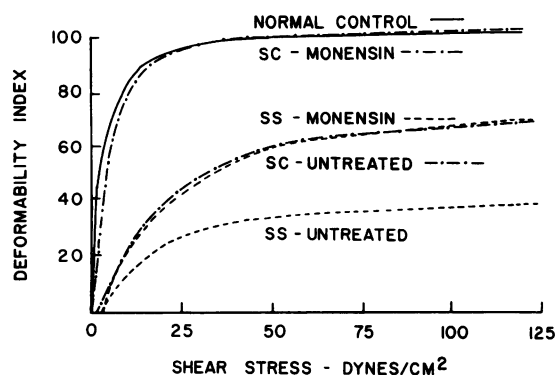


FIGURE 4 DI vs. shear stress for SC and SS cells; both untreated, and treated with 10 nM Monensin in the absence of plasma. Note the virtual normalization of the SC deformability curve as a result of Monensin treatment.

ity. This suggests that the difference can be explained on the basis of a greater uniformity in the extent of dehydration in the SC samples than in the SS samples.

## DISCUSSION

In this study we have tested the hypothesis that a controlled increase in erythrocyte membrane permeability might provide an effective means of increasing the water content of sickle cells. Monensin, an antibiotic that has activity as a Na-selective ionophore, was effective at submicromolar concentrations in producing sustained hydration of sickle cells without causing hemolysis. Both discoid sickle cells and ISC increased their water content as a result of Na uptake, and the consequent reduction in MCHC improved the deformability of ISC-rich cell populations and of unseparated sickle cells. It is anticipated that the reduction in HbS concentrations within the entire cell population should confer substantial resistance to sickling, perhaps preventing the process of sickling-induced dehydration that is believed to accompany ISC formation.

Results of the experiments in which cells were treated with Monensin in the presence of DIDS agree with previous conclusions about the mechanism of Monensin action (10). In the presence of a suitable Na or pH gradient, Monensin will exchange a Na ion for a proton. When Monensin is added under physiological conditions, the gradients across the erythrocyte membrane drive Na into the cell in exchange for intracellular protons. If there were no means of relaxing the pH gradient that would build up from this exchange, the extent of ion exchange would be limited. In the erythrocyte, however, hydroxyl ions can exchange for chloride ions through the anion channel. Thus, while Monensin facilitates the exchange of extracellular Na ions for intracellular protons, the anion channel per-

mits the simultaneous exchange of intracellular hydroxyl ions for extracellular chloride ions. The two exchanges together result in an influx of NaCl and an efflux of an equivalent amount of water. However, the efflux of water mediated by the ion exchange processes is totally overwhelmed by a much larger (more than two orders of magnitude) influx of osmotically driven water. In our experiments, inclusion of DIDS with Monensin blocked the hydroxyl/chloride exchange, preventing relaxation of the pH gradient (14). The net influx of NaCl was thereby limited, and cell swelling did not occur.

Other methods of decreasing MCHC have been suggested, such as promotion of iron deficiency (16) and reduction in plasma Na concentration and osmolality (7). The latter strategy has been tested clinically through administration of antidiuretic hormone and limitation of dietary Na (7-9). Although an initial report suggested that this was an effective therapy for prevention of sickling crises (7), subsequent studies have achieved less satisfactory results (8, 9). Other investigators have also explored the possibility of increasing sickle cell Na content as a means of reducing the intracellular concentration of HbS. Orringer et al. (17) employed the ingenious approach of smuggling Na through the anion channel as  $\text{NaCO}_3^-$  ion. These experiments required the use of high concentrations of bicarbonate in vitro, and after reinfusion into the blood donor, the cells lost their excess Na and water within a day or so. Chang and Nagel have more recently evaluated several agents for their utility in hydrating sickle cells, also in an extracorporeal system (18). Their observations suggested that gramicidin might be a useful agent for this approach, although its toxicity would limit its use to in vitro treatment of cells. Obviously, a drug that requires extracorporeal treatment would have limited usefulness, and we are primarily interested in drugs that could provide effective hydration of cells in vivo. Monensin itself may not be the appropriate drug for this purpose. When given to experimental animals, it becomes widely distributed throughout body tissues and causes an elevation in heart rate and blood pressure at plasma concentrations in the micromolar range (19). At present it is not clear whether erythrocyte cation content is altered at drug concentrations in vivo below the threshold for deleterious effects on other tissues. If Monensin itself turns out to be too toxic to use, it might be possible to modify the drug by either pharmacologic or immunologic manipulations to give it a higher affinity for the erythrocyte membrane, perhaps a selective affinity that could prolong its action and limit such possible toxicity.

These experiments represent an early stage in the evaluation of a therapeutic approach to sickle cell dis-

ease that is based on increasing membrane permeability for Na. Many questions remain to be addressed, but Monensin's potent effects on both MCHC and ISC deformability provide encouragement for this approach.

#### ACKNOWLEDGMENTS

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