

Evidence for a Direct Reticulocyte Origin of Dense Red Cells in Sickle Cell Anemia

Robert M. Bookchin, Olga E. Ortiz, and Virgilio L. Lew*

Department of Medicine, Albert Einstein College of Medicine, Bronx, New York 10461; and

*Physiological Laboratory, Cambridge University, Cambridge, United Kingdom

Abstract

To explore our hypothesis of a direct reticulocyte origin of irreversibly sickled cells (ISCs), we fractionated light, reticulocyte-rich, and discocyte-rich sickle cell anemia red cells on Stractan gradients, and examined the effects of deoxygenation-induced sickling, external Ca^{2+} , acidification, and replacing external Na^+ by impermeant *N*-methyl-D-glucamine (NMG^+). Sickling permeabilized light reticulocyte-rich cells to cations (Na^+ , K^+ , and Ca^{2+}) more than discocytes; without external Ca^{2+} , Na^+ influx matched K^+ efflux, with stable cell volume; with Ca^{2+} , many light, low hemoglobin (Hb) F reticulocytes dehydrated rapidly (preventable by quinine, a Ca^{2+} -dependent K^+ channel inhibitor). Acidification of oxygenated discocytes (high mean Hb F) and reticulocyte-rich fractions yielded denser, reticulocyte-enriched cells with lower Hb F (as in light reticulocyte or dense ISC-rich fractions). Light cells shrank when NMG^+ replaced Na^+ , supporting predictions of a Na^+ -dependent volume control system. Demonstration of sickling-induced, Ca^{2+} -dependent dehydration of Hb F-free reticulocytes, and conservation of acid-stimulated $\text{K}:\text{Cl}$ cotransport among low Hb F, reticulocyte-enriched cells in discocyte fractions support the hypothesis. Ancillary new findings included heparin stimulation of sickling-induced Na^+ and K^+ permeabilizations, and Ca^{2+} inhibition of the Na^+ leak. (*J. Clin. Invest.* 1991. 87:113–124.) Key words: sickle cell anemia • reticulocyte • red cell dehydration • ion transport • Ca^{2+}

Introduction

The previous paper (1) described a new non-steady-state mathematical model of the volume, pH, and ion content regulation of reticulocytes. The model incorporates the volume- and pH-sensitive $\text{K}:\text{Cl}$ cotransport features recently documented in the light, reticulocyte-enriched fractions of sickle cell anemia (SS)¹ red blood cells, (RBCs) (2–4). On the basis of the theoretical predictions of that model, together with the previous documentation of the young age of dense irreversibly

sickled cells (ISCs) (5) and of RBC acidification following K^+ permeabilization (6), we developed a new hypothesis on the mechanism of formation of dense SS RBCs. According to this hypothesis (1, 7), most ISCs and other dense SS RBCs are primarily generated directly from a fraction of reticulocytes rather than by progressive dehydration of mature discocytes.

The experiments described in this article were designed with the following purposes: (a) to test the feasibility of one of the two basic mechanisms for SS RBC dehydration within the above hypothesis, by determining whether deoxygenation and sickling result in cation (Na^+ , K^+ , Ca^{2+}) permeabilization of the light, reticulocyte-rich fraction of SS cells, and can thereby act as an initial trigger or a part of the positive feedback mechanism for progressive dehydration of the reticulocytes; (b) to examine the effects of any such permeabilization on the volume and density of the cells; (c) to test the model's predicted effects of changes in medium pH and Na^+ concentration; (d) to search for the predicted presence of SS RBCs with transport properties of reticulocytes in various density fractions; and (e) to examine whether the experimental procedures that result in dehydration of SS RBC in vitro alter the cell density distribution of hemoglobin (Hb) F (which is known to inhibit cell sickling), and thereby help determine the relevance of sickling in SS RBC dehydration in vivo.

Methods

Composition of solutions. The solutions used contained (in mM): Solution A, 75 NaCl; 75 KCl; 10 Na-Hepes (pH 7.5); 0.1 Na-EGTA. Solution B, same as solution A but without Na-EGTA. Solution C, 90 KCl; 60 NaCl; 10 Na-Hepes (pH 7.5); 0.2 MgCl_2 ; 0.02 Na-EGTA. Solution D, 5 KCl; 145 NaCl; 10 Na-Hepes (pH 7.4 or 7.0); 0.2 MgCl_2 ; 0.04 Na-EGTA. Solution E, 108 MgCl_2 ; 10 Tris-Cl (pH 7.4 at 20°C). "Glucamine medium," 5 KCl; 145 *N*-methyl-glucamine (NMG^+)-Cl; 10 Tris-Cl; 0.04 Tris-EGTA (either at pH 7.4 or 7.0 at 37°C).

Preparation of cells. Heparinized venous blood was obtained after informed consent from sickle cell anemia patients with established genotype, and kept at 4°C for no more than 2 h before processing. The plasma was separated from the cells after centrifugation at 2,500 *g* for 10 min. When needed for later cell resuspension, plasma was spun further at 30,000 *g* for 40 min at 4°C, decanted, and, after adding 10 mM inosine and 1 mM adenine, filtered through cellulose acetate membranes with 0.45- μm pores (Nalge Co., Rochester, NY) and kept on ice until use. The packed cells were filtered through a nylon mesh (100 \times polyamide nylon fiber, mesh 20 μm , Nitex; Tetko Inc., Elmsford, NY) to remove white cells without loss of reticulocytes, washed in solution A, filtered twice more, and finally washed and resuspended in the appropriate incubation medium, or in autologous plasma.

For experiments in heparin-free serum, blood was collected in a syringe containing Na-EGTA (pH 7.4) to give a final concentration of 3.2 mM in the plasma. The plasma was removed and, after addition of 3.2 mM CaCl_2 , defibrinated by swirling in a flask containing glass beads before centrifugation and further handling as above.

Density separation procedure. SS RBCs suspended at 40% hematocrit in solution A were layered onto discontinuous gradients of arabinogalactan (Stractan II; St. Regis Paper Co., Libby, MT; referred to in

A preliminary report of portions of this work was published in abstract form (1988. *Clin. Res.* 36:613A).

Address correspondence to Dr. Robert M. Bookchin, Department of Medicine, Rm. 913U, Albert Einstein College of Medicine, 1300 Morris Park Avenue, Bronx, NY 10461.

Received for publication 28 August 1989 and in revised form 12 June 1990.

1. Abbreviations used in this paper: Hb, hemoglobin; ISC, irreversibly sickled cell; NMG^+ , *N*-methyl-D-glucamine; RBC, red blood cell; SS, homozygous hemoglobin S disease, or sickle cell anemia.

J. Clin. Invest.

© The American Society for Clinical Investigation, Inc.

0021-9738/91/113/12 \$2.00

Volume 87, January 1991, 113–124

the text below as Stractan) using a modification (8) of the method of Corash et al. (9). The densities varied with the experimental protocols, as noted below. The cells with $1.000 < \delta \leq 1.087$ always had the highest percentage of reticulocytes (as measured by standard procedures after supravital staining with brilliant cresyl blue) and $< 1\%$ white cell contamination. In many experiments a second density layer with δ between 1.087 and 1.091 was also used as a reticulocyte-rich cell fraction, and additional, higher density layers were used as indicated below to isolate the discocyte-rich cell fractions. The cell fractions were washed three times in solution B and, for experiments done the same day, washed once more and resuspended in the final incubation medium. Alternatively, the cell fractions were kept overnight suspended in solution C at 4°C , and before experiments were washed and resuspended in solution D and incubated for 1 h at 37°C . Preliminary experiments indicated that after this procedure the cell ATP content was normal and the cell density and Na^+ and K^+ content were essentially the same as they were just after density separation. Finally, the cells were washed three times in solution B and washed once and resuspended in their incubation medium.

The phthalate ester method of Danon and Marikovsky (10) was used, with minor modifications, to compare RBC density distributions before and after certain experimental protocols. Aliquots of RBC suspension were transferred to microcapillary tubes containing mixtures of diethylphthalate and dibutylphthalate with densities ranging from 1.042 to 1.117. After 2 min centrifugation at 12,000 rpm in a microhematocrit centrifuge the Hb content of the pellet was measured and expressed as a percentage of the total Hb in the aliquot.

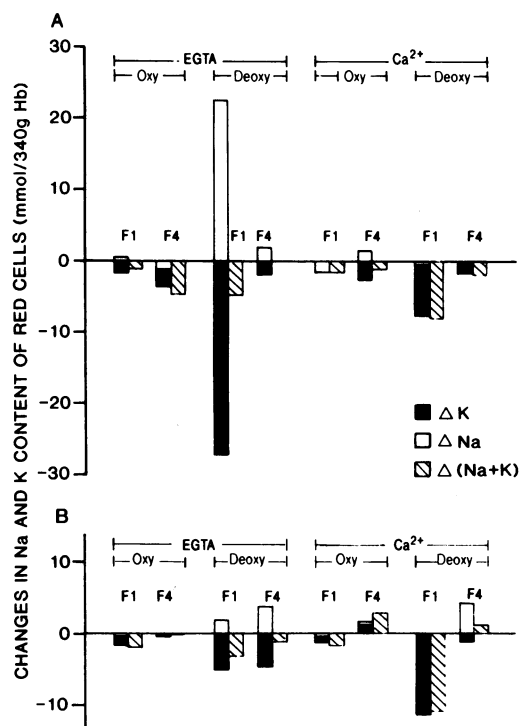


Figure 1. Effect of deoxygenation on the Na^+ and K^+ content of SS cell density fractions with different proportions of reticulocytes. Final cell suspension in autologous serum (10% Hct), either with normal serum Ca^{2+} levels or containing 3 mM of EGTA (neutralized with NaOH) to reduce $[\text{Ca}^{2+}]_0$ to submicromolar levels. *A* and *B* correspond to experiments with two different donors, and further details are summarized in Table I. *A* corresponds to donor J.E. in Table I (three density fractions, suspended in serum), and *B* corresponds to donor A.F. The percent reticulocytes in each fraction are indicated in the table. For both donors the F1 fraction cells had density $\delta \leq 1.087$, and for F4 $1.095 < \delta \leq 1.106$.

Deoxygenation of RBCs. RBCs suspended at 10% Hct in autologous plasma, serum, or bicarbonate-containing buffers were equilibrated in a tonometer (model 237; Instrumentation Laboratory Inc., Lexington, MA) at 37°C with humidified N_2 or argon containing 5.6% CO_2 . Gases without CO_2 were used for RBC suspensions in nonbicarbonate buffers. The suspension pH levels were monitored at 37°C (pHM 71 Mk2, and G297/G2 Capillary Electrode; Radiometer, Copenhagen, Denmark) and adjusted to 7.40–7.45 by addition of 150 mM HCl or NaHCO_3 .

Measurement of RBC content of Na^+ and K^+ . RBCs were washed three times with ice-cold solution E, packed, and lysed in 15 mM LiCl. The Hb concentration was measured (as cyanmet-Hb) on an aliquot of the lysate, and the Na^+ and K^+ concentrations were determined by flame photometry (IL model 143; Instrumentation Laboratory Inc.) and expressed as millimoles per 340 g Hb.

Measurement of calcium influx into chelator-loaded RBCs. RBCs were preloaded with the high-affinity Ca^{2+} chelator, Benz2, by the method of Lew et al. (11), in the presence of 5 mM pyruvate to prevent ATP depletion (12, 13). The intracellular chelator reduces Ca^{2+} below levels that activate the Ca^{2+} pumps (11), so that the rate of increase in total cell calcium can serve as a measure of passive calcium influx with minimal interference from Ca^{2+} pump extrusion fluxes. The method of Tiffert et al. (12) was used to estimate chelator incorporation and measure influx of ^{45}Ca .

Measurement of Hb F. The percentage of Hb F in RBC was estimated, after electrophoresis of the hemolysates on citrate agar gels at pH 6.3 (Corning Medical and Scientific, Palo Alto, CA), by densitometry of the unstained gels. All measurements were made in triplicate or more (except where duplicates were identical to the first decimal) and showed a mean standard error of 0.65% Hb F.

Examination of RBC morphology. For examination of RBC morphology before and after various experimental procedures, cell samples were fixed in 10% formalin in phosphate-buffered (pH 7.4) isotonic saline and later examined with a microscope (Carl Zeiss, Inc., Thornwood, NY) equipped with Nomarski optics.

The percentage of reticulocytes in original blood samples and in density fractions of RBCs before and after incubations was measured by the standard technique of microscopic examination of RBC smears after supravital staining with brilliant cresyl blue.

Results

Effects of deoxygenation in the presence and absence of external Ca^{2+} on the Na^+ and K^+ content of SS cell fractions. The two experiments in Fig. 1 compare the extent of sickling-induced Na^+ and K^+ permeabilization in the presence and absence of physiological levels of external Ca^{2+} with two SS cell density fractions: the lightest fraction, highly enriched in reticulocytes; and a fraction with density similar to most normal RBCs, containing much lower proportions of reticulocytes.

During the 30-min incubation at 37°C in autologous serum, with added EGTA to chelate all external Ca^{2+} , the light, reticulocyte-rich SS cells (F1) in the experiment in Fig. 1 *A* showed a large deoxygenation-related increase in Na^+ and K^+ leaks, with a small mean net K^+ loss over Na^+ gain. Much smaller, balanced Na^+ and K^+ leaks occurred during deoxygenation of the discocyte-rich (F4) fraction. Qualitatively similar results were seen with blood from another donor, shown in Fig. 1 *B*, where the fluxes induced by deoxygenation of the reticulocyte-rich cells (in the absence of external Ca^{2+}) were smaller, and differed little from those of the discocyte fraction. Other similar experiments showed only small flux differences in conditions with and without ouabain, indicating little influence of the sodium pump on these net cation shifts.

In the presence of physiological serum Ca^{2+} levels, deoxy-

Table I. Effect of External Ca^{2+} on the Deoxygenation-induced Changes in the Na and K Content of Density-fractionated SS RBCs Suspended in Serum or Buffers

Donor	Density fraction	Retics	Na and K content of RBCs							
			Changes after 30 min deoxygenation							
			Initial values		Excess EGTA			1.2 mM Ca ²⁺		
			Na	K	ΔNa	ΔK	Δ(Na + K)	ΔNa	ΔK	Δ(Na + K)
			mmol/340 g Hb							
J.N.*	δ ≤ 1.087	53	29.7	126	+5.7	-10.9	-5.2	+2.6	-11.6	-9.0
T.N.*	1.087 < δ ≤ 1.091	70	17.4	114	+6.9	-10.7	-3.8	+7.0	-10.3	-3.3
J.E. [‡]	δ ≤ 1.087	60	9.5	148	+1.9	-8.3	-6.4	-1.5	-2.9	-4.4
J.E. [§]	δ ≤ 1.087	60	9.5	148	+1.9	-7.6	-5.7	-1.8	-9.1	-10.9
B.W. [§]	δ ≤ 1.091	57	9.7	138	+18.7	-20.7	-2.0	+11.8	-21.9	-10.1
A.F.	δ ≤ 1.087	50	14.7	132	+1.7	-4.9	-3.5	+0.3	-10.9	-10.6
	1.087 < δ ≤ 1.091	26	34.1	105	-0.1	-2.4	-2.5	+4.2	-2.8	+1.4
	1.095 < δ ≤ 1.106	5	12.8	96	+3.7	-4.6	-0.9	+3.4	-1.0	+2.4
J.E.	δ ≤ 1.087	64	10.0	129	+22.5	-27.0	-4.5	-0.4	-7.5	-7.9
	1.087 < δ ≤ 1.091	39	8.3	108	+3.9	-4.5	-0.6	+1.3	-0.3	+1.0
	1.095 < δ ≤ 1.106	20	13.7	87	+1.9	-1.9	0.0	-0.4	-1.6	-2.0

SS RBCs with the densities shown were suspended in autologous serum or buffer as noted at 10% Hct, with or without added EGTA (to give nominally Ca^{2+} -free plasma). For cell suspensions in buffers, sufficient CaCl_2 was added to give ~ 1.2 mM Ca^{2+} in the presence of the corresponding amounts of albumin. The mean content of RBC Na and K in each sample was measured before and after incubation at 37°C with 30 min deoxygenation and 5 min reoxygenation. Gases for deoxygenation and reoxygenation of serum and bicarbonate buffers contained 5.6% CO_2 . * Suspended in buffer containing 5.0 g/dl of bovine serum albumin and (in mM) 5 KCl, 145 NaCl, 10 Na-HEPES (pH 7.40), 1.0 adenosine, 10 inosine, 10 glucose, and 0.2 MgCl_2 (adjusted to 300 mosmol/kg H_2O). † Suspended in buffer containing 4.0 g/dl of bovine serum albumin and (in mM) 5 KCl, 127 NaCl, 1.0 MgCl_2 , 1.2 NaH_2PO_4 , (total osmolality 299 mosmol/kg H_2O). ‡ Suspended in buffer of the same composition as † (above) except that 27 mmol/liter of NaCl was replaced by equimolar NaHCO_3 . ¶ Suspended in serum.

Table II. Effect of External Ca^{2+} on the Deoxygenation-induced Changes in the Na and K Content of Density-fractionated SS RBCs Suspended in Heparinized Plasma

			Na and K content of RBCs							
Donor	Density fraction	Retics	Changes after 30 min deoxygenation							
			Initial values		Excess EGTA			1.2 mM Ca ²⁺		
			Na	K	ΔNa	ΔK	Δ(Na + K)	ΔNa	ΔK	Δ(Na + K)
			mmol/340 g Hb							
		%								
M.M.	δ ≤ 1.091	78	15.6	143	+11.1	-11.9	-0.8	+4.4	-7.6	-3.2
M.M.	δ ≤ 1.091	63	9.7	124	+8.0	-8.8	-0.8	+3.7	-8.3	-4.6
M.M.	δ ≤ 1.087	—	13.3	125	+8.2	-9.5	-1.3	+3.3	-8.1	-4.8
M.M.	1.087 < δ ≤ 1.091	50	9.0	122	+4.1	-6.6	-2.5	-0.7	-1.0	-1.7
J.E.	δ ≤ 1.087	56	9.6	142	+16.4	-17.1	-0.7	+8.1	-20.3	-12.2
J.E.	δ ≤ 1.087	53	12.5	135	+18.1	-16.6	+1.5	+15.2	-21.4	-6.2
J.E.	δ ≤ 1.087	60	9.5	148	+13.1	-20.9	-7.8	+2.0	-2.6	-0.6
T.N.	1.087 < δ ≤ 1.091	70	17.4	114	+24.1	-23.3	+0.8	+10.1	-16.4	-6.3
K.W.	δ ≤ 1.091	64	20.1	122	+45.7	-47.9	-2.2	+27.7	-33.8	-6.1
B.W.	δ ≤ 1.091	57	9.7	138	+53.9	-54.2	-0.3	+14.6	-20.2	-5.6
A.F.	δ ≤ 1.087	67	21.7	124	+15.9	-19.1	-3.2	+2.1	-4.7	-2.6
	1.087 < δ ≤ 1.091	47	9.4	115	+7.9	-6.1	+1.8	+2.8	-3.5	-0.7
	1.091 < δ ≤ 1.095	9	13.0	96	+5.1	-5.3	-0.2	+5.3	-5.2	+0.1

SS RBCs with the densities shown were suspended in autologous plasma at 10% Hct with or without added EGTA (to give nominally Ca^{2+} -free plasma). The mean content of RBC Na and K in each sample was measured before and after incubation at 37°C with 30 min deoxygenation and 5 min reoxygenation.

generation of the reticulocyte-rich (F1) fractions in both experiments of Fig. 1 induced potassium leaks with little or no sodium leaks, so that mean net cation losses predominated. In the experiment in Fig. 1 *A* it is not possible to distinguish whether the net K^+ loss in the presence of Ca^{2+} resulted from activation of the K^+ (Gardos) channel or from greater inhibition of the Na^+ leak than the K^+ leak induced by deoxygenation. In the Fig. 1 *B* experiment, however, the K^+ leak on deoxygenation of the light cell fraction was actually larger in the presence of Ca^{2+} , suggesting K^+ channel activation.

Table I shows the details of several experiments similar to (and including) those in Fig. 1, with the RBCs incubated in either serum or albumin-containing buffers. In most instances, the mean net cation shifts included smaller Na^+ gains and larger K^+ losses when external Ca^{2+} was present. Experiments with one donor (J.E.) showed no differences in the sickling-induced fluxes in the presence or absence of bicarbonate.

Interrelated effects of heparin and Ca^{2+} on sickling-induced Na and K leaks. The experiments in Table II are grouped together because they were all done with RBCs suspended in plasma containing heparin. In these conditions, at very low external Ca^{2+} (with excess EGTA), the deoxygenation-induced changes in Na^+ and K^+ content after 30 min were generally much larger than those in the absence of heparin (only with the light, reticulocyte-rich fractions), and fairly balanced. Other experiments (not shown here since they are part of a separate study) indicated that heparin per se stimulated the sickling-induced Na^+ and K^+ leaks in this cell fraction. External Ca^{2+} reduced the deoxygenation-induced leaks of both Na^+ and K^+ but, as in the heparin-free experiments, Na^+ gain was inhibited considerably more than K^+ loss. Therefore, the presence of physiological Ca^{2+} concentrations in the medium resulted in significant net cation loss despite the lower overall magnitude of the deoxygenation-induced leaks. A control experiment

Table III. Effects of External Ca^{2+} and Dexoxygenation of the Reticulocyte-rich Fraction of SS RBCs on Their Density Distribution and Na and K Contents

Donor	Retics	Stractan density (δ)	Initial values			After 30 min deoxygenation					
			RBC with $\delta > \text{Stractan}$	Na		Excess EGTA			1.2 mM Ca^{2+}		
				mmol/340 g Hb	K	RBC with $\delta > \text{Stractan}$	Na	K	RBC with $\delta > \text{Stractan}$	Na	K
	%		%	mmol/340 g Hb		%	mmol/340 g Hb		%	mmol/340 g Hb	
A.F.*	67	1.000	100	21.7	124	100	37.6	105	100	20.8	116
		1.074	92	13.2	126	77	21.8	114	92	14.8	117
		1.083	71	9.3	126	53	14.5	11	70	11.1	116
		1.087	49	9.1	125	35	13.0	120	52	11.4	112
		1.091	8	7.1	118	7	11.2	115	24	14.7	103
		1.095	0	—	—	0	—	—	5	30.3	83
K.W.*	64	1.000	100	19.2	128	100	64.9	75	100	48.6	83
		1.074	89	12.1	122	63	33.8	81	89	45.1	—
		1.083	73	9.4	—	49	29.6	96	83	44.2	89
		1.087	24	8.5	102	13	27.9	77	61	45.9	65
		1.091	0	—	—	2	—	—	50	47.1	61
		1.095	0	—	—	0	—	—	38	47.4	64
J.E.*	60	1.000	100	9.5	148	100	22.5	127	100	11.4	145
		1.079	82	9.0	142	82	15.0	126	89	10.6	139
		1.083	38	7.6	133	52	14.2	126	64	11.5	129
		1.087	0	—	—	20	17.1	121	27	13.9	114
		1.091	0	—	—	6	—	—	15	17.9	109
J.E.†	60	1.000	100	9.5	148	100	11.4	141	100	9.6	139
		1.079	82	9.0	142	84	10.8	137	92	9.8	134
		1.083	38	7.6	133	63	9.3	134	65	9.7	132
		1.087	0	—	—	23	8.4	131	32	10.0	128
		1.091	0	—	—	4	—	—	17	10.4	121
B.W.*	57	1.000	100	9.7	138	100	63.6	83	100	24.3	117
		1.079	100	9.7	138	71	31.6	111	100	24.3	117
		1.091	9	—	—	16	—	—	50	28.2	99
B.W.†	57	1.000	100	9.6	138	100	28.4	122	100	21.5	116
		1.079	100	9.6	138	82	17.3	134	98	18.4	119
		1.091	5	—	—	19	—	—	46	27.3	108

SS RBCs from each of the density fractions shown were suspended at 10% Hct in the media shown with or without added EGTA (to give nominally Ca^{2+} -free plasma). See legend of Table I for addition of calcium to buffers. The mean content of RBC Na and K in each sample was measured in each density fraction before and after incubation at $37^\circ C$ with 30 min deoxygenation and 5 min reoxygenation. * Suspended in heparinized plasma. † Suspended in buffer containing 4.0 g/dl bovine serum albumin and (in mM) 5 KCl, 100 NaCl, 27 $NaHCO_3$, 1.0 $MgCl_2$, 1.2 NaH_2PO_4 , (total osmolality 299 mosmol/kg H_2O).

(not shown) in which Ca-EGTA was added to the plasma, leaving normal plasma Ca^{2+} , showed no difference from the results with plasma Ca^{2+} alone, thereby excluding the possibility that the effect of EGTA was due to chelation of trace amounts of another heavy metal.

The effects of Ca^{2+} on the sickling-induced leaks are more evident in Table III, which shows the cation contents of each of the new density fractions formed after deoxygenation of the original light, reticulocyte-rich SS RBCs. In each experiment

(with or without heparin), Ca^{2+} induced the formation of large numbers of denser cells, and these newly dense cells showed the most dramatic net K^+ loss, exceeding their Na^+ gain.

In all of these experiments there were considerable variations between donors in the size of the net cation shifts, and sometimes in different experiments with the blood samples from the same donor.

Effect of deoxygenation on the calcium uptake by chelator-loaded SS cells. The uptake of Ca^{2+} by SS discocytes and by reticulocyte-rich SS cell fractions was investigated after loading the cells with a chelator to maintain cytoplasmic Ca^{2+} below levels that activate the calcium pump. In this condition Ca^{2+} influx measures passive inward leak. Fig. 2 shows that, in agreement with early studies (14), passive calcium uptake in oxygenated reticulocytes is higher than in discocytes, and that

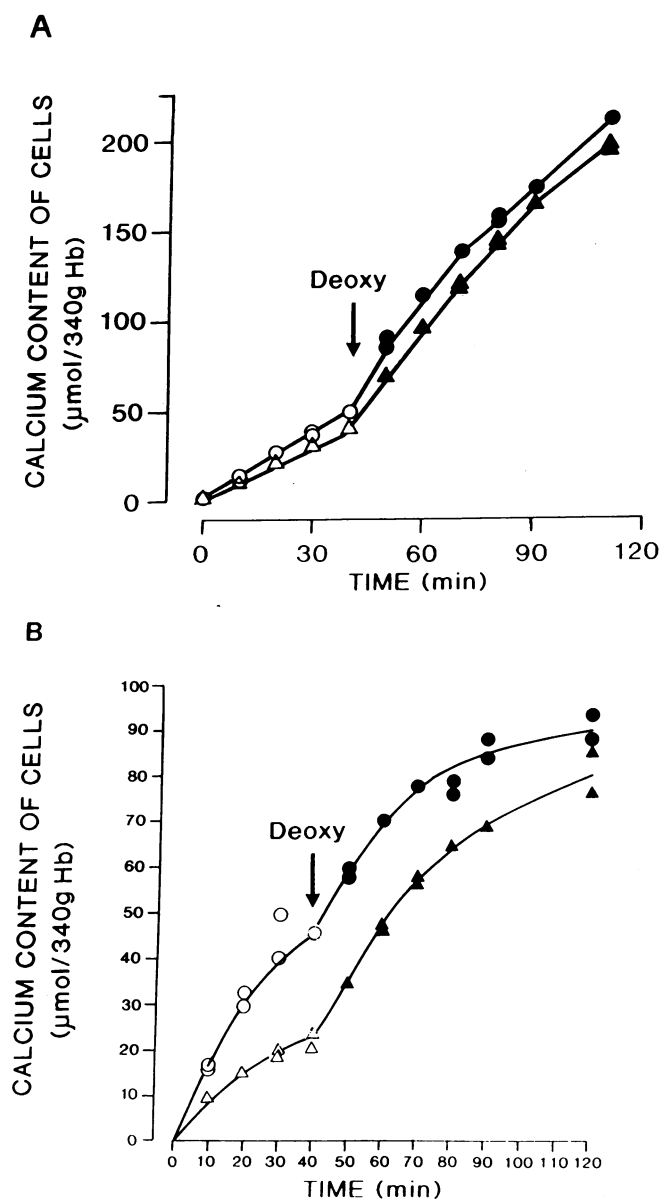


Figure 2. Effect of deoxygenation on ^{45}Ca uptake by density fractions of SS RBCs loaded with the calcium chelator Benz2. The RBCs were suspended at 10% Hct in autologous plasma. Each panel shows an experiment with RBCs from a separate donor, and two density fractions, $1.087 < \delta < 1.091$ (circles) and $1.095 < \delta \leq 1.106$ (triangles). The percentages of reticulocytes in the lighter and heavier fractions were 28 and 2%, respectively, in A, and 64 and 10% in B. Intracellular Benz2 concentrations, in millimoles/liter of cells, were 4.2 and 3.6, respectively, for reticulocytes and discocytes in the experiment in A, and 0.23 and 0.074 in the experiment in B.

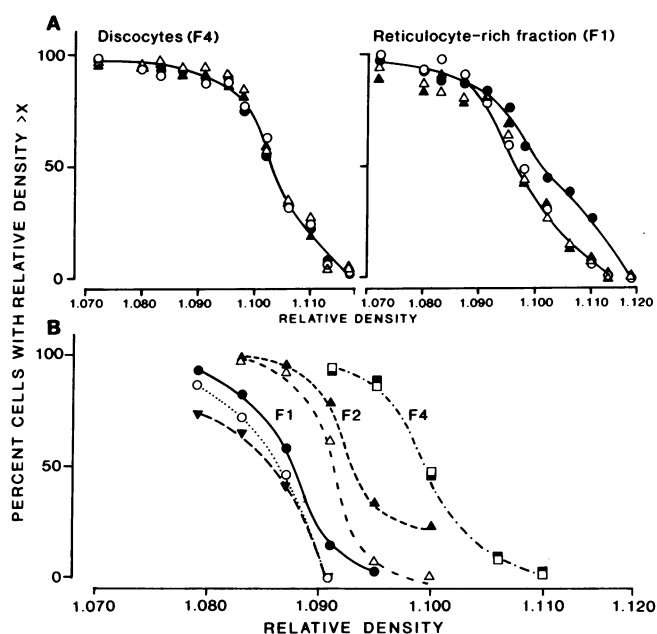


Figure 3. Effect of deoxygenation and external Ca^{2+} on the density distribution of SS discocytes and light, reticulocyte-rich fractions of SS RBCs. (A) This experiment used two fractions of SS RBCs obtained from Stractan gradients: F1, a fraction with density $\delta \leq 1.087$, containing 61% reticulocytes; and F4, a discocyte-rich fraction with density $1.095 < \delta \leq 1.105$, containing 18% reticulocytes. The RBCs were suspended at 20% Hct in autologous heparinized plasma, with or without addition of 3 mM EGTA, and equilibrated in a tonometer at 37°C with the appropriate gas mixture (see Methods); for this experiment, "oxy" equilibration was in 5.6% CO_2 in oxygen, and "deoxy" equilibration was with the above oxygen mixture for 5 min alternating with 5.6% CO_2 in argon for 20 min. After 55 min incubation the density distribution of each RBC suspension was measured using phthalate esters (see Methods). Δ , oxy, EGTA; \blacktriangle , deoxy, EGTA; \circ , oxy, Ca^{2+} present; \bullet , deoxy, Ca^{2+} present. (B) Three Stractan density fractions of SS RBCs were used in this experiment: F1, with $\delta \leq 1.087$ and 64% reticulocytes; F2, $1.087 < \delta \leq 1.091$, with 39% reticulocytes; and F4, $1.095 \leq \delta < 1.106$, with 20% reticulocytes. The RBCs were suspended in autologous serum and the incubations were performed as in A (above) except that the period of deoxy incubation was 30 min. The density distribution of each suspension was redetermined using the same Stractan solutions as for the initial separations. For F1: \circ , initial; \bullet , deoxy, Ca^{2+} present; ∇ , deoxy, EGTA; for F2: Δ , initial; \blacktriangle , deoxy, Ca^{2+} present; for F4, \square , initial; \blacksquare , deoxy, Ca^{2+} present.

deoxygenation markedly increases calcium influx. Thus, as with SS discocytes, the deoxygenation-induced increase in SS reticulocyte permeability apparently affects at least Na^+ , K^+ , and Ca^{2+} , and may be nonselective to small ions in general.

Effects of deoxygenation and external Ca^{2+} on the density distribution of SS discocytes and reticulocytes. The experiments of Fig. 3 show that when SS discocytes and reticulocyte-rich cells were suspended in either autologous heparinized plasma or autologous serum and incubated at 37°C for 30–55 min, an increase in cell density was seen only with the light, high reticulocyte cell fractions, and only when the cells were deoxygenated in the presence of Ca^{2+} . There was a consistent enrichment in the proportion of reticulocytes among the cells that became dense when deoxygenated in the presence of Ca^{2+} . This is seen in a similar experiment in Table IV, which shows the effects of external Ca^{2+} and of deoxygenation on the density and reticulocyte redistribution of a light, reticulocyte-rich SS RBC fraction. Upon incubation in serum for 30 min, an increase in cell density occurred in a substantial fraction of cells only in the deoxygenated condition with physiological levels of serum Ca^{2+} , and nearly all the cells that increased in density were reticulocytes.

In a separate, similar experiment (not shown), after deoxygenation of a reticulocyte-rich fraction of SS RBCs ($\delta < 1.087$) for 30 min in the presence of Ca^{2+} , the morphology of the newly dehydrated cells was examined after further density fractionation. Each of these fractions showed an increase in the proportion of cells with the convoluted membrane surfaces typical of “stress reticulocytes” (from 13% of the original light cells to 23–33% of denser fractions), and an increased number of small, deformed cells typical of dense non-ISCs from fresh SS blood. But a small fraction, comprising the densest of the newly dehydrated cells ($\delta > 1.100$), showed $\sim 20\%$ of cells whose length was at least twice their width, and had the appearance of relatively short, fat ISCs.

These results show that, in contrast to the behavior of SS discocytes, many of the cells in the light, reticulocyte-rich SS

fractions dehydrate rapidly when deoxygenated in the presence of plasma levels of Ca^{2+} . Most of these newly dehydrated cells are reticulocytes, including an enrichment in “stress reticulocytes,” but the fraction that becomes dense and the proportion of reticulocytes within it vary from patient to patient. Even after brief incubation under these conditions, a small number of cells meet the morphological criteria of ISCs.

Effects of quinine on the changes in density of reticulocyte-rich SS cell fractions induced by deoxygenation in the presence and absence of Ca^{2+} . To help discern a possible role of the Ca^{2+} -dependent K^+ channel in unbalancing the Na^+ and K^+ leaks when reticulocyte-rich SS cell fractions were deoxygenated in the presence of external Ca^{2+} , we examined the effects of addition of quinine, a well characterized (although nonspe-

Table IV. Effects of External Ca^{2+} and Deoxygenation on the Density and Reticulocyte Redistribution of a Light, Reticulocyte-rich Fraction of SS RBCs

Sample	$\delta < 1.087$		$\delta > 1.087$	
	Percent of total RBC	Percent reticulocytes	Percent of total RBC	Percent reticulocytes
Initial	100	46	0	—
Oxy + EGTA	99	39	1	100
Deoxy + EGTA	99	48	1	100
Oxy + Ca^{2+} *	99	39	1	100
Deoxy + Ca^{2+} *	90	46	10	95

A fraction of RBCs with density $\delta \leq 1.087$, containing $\sim 45\%$ reticulocytes, was suspended at 20% Hct in autologous serum with or without addition of 3 mM EGTA. After 30 min incubation at 37°C in either 5.6% CO_2 with oxygen (oxy) or argon (deoxy) 0.5 ml of the suspension was layered onto a discontinuous gradient of seven density layers of Stractan to determine the RBC density distribution. Only after the Deoxy + Ca^{2+} incubation did some cells (reticulocytes) achieve densities greater than 1.095 and 1.100.

* Original serum levels of Ca^{2+} .

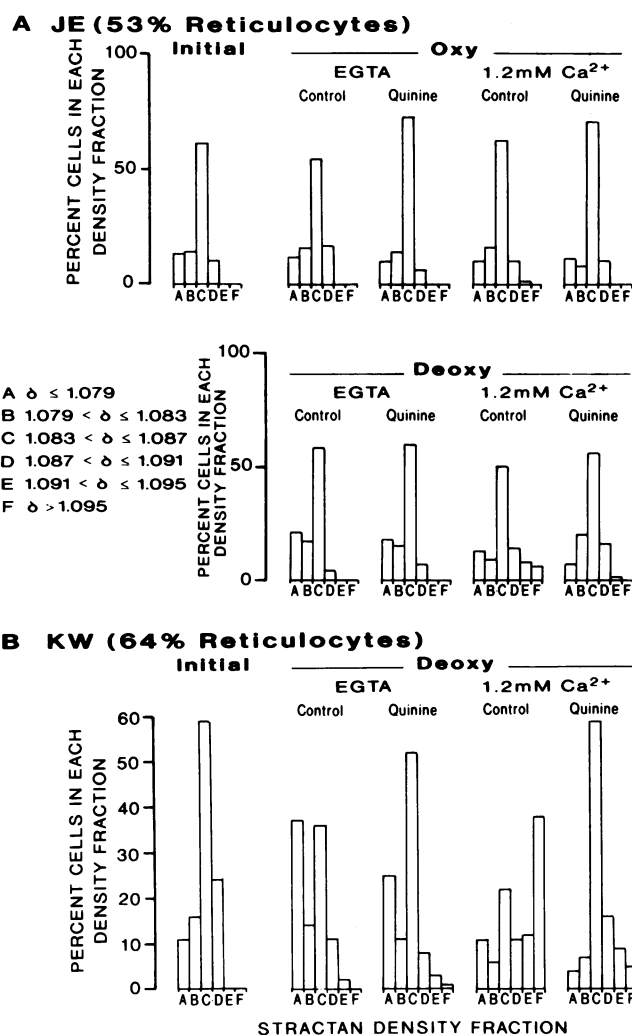


Figure 4. Effects of quinine, Ca^{2+} , and deoxygenation on the density redistribution of a light, reticulocyte-rich fraction of SS RBCs. Two experiments are shown in which RBC fractions with density $\delta \leq 1.087$ were suspended at 20% Hct in autologous plasma with or without addition of 3 mM EGTA and with or without 1 mM quinine hydrochloride. After 30 min incubation at 37°C in either 5.6% CO_2 with oxygen (oxy) or argon (deoxy), 0.5 ml of the suspension was layered onto a discontinuous gradient of Stractan (densities shown), and the percentage of cells (measured as percent Hb) in each density fraction was determined.

cific) inhibitor of Ca^{2+} -activated K^+ channels in red cells (15). As shown in the two experiments in Fig. 4, with oxygenated cells with or without Ca^{2+} , or with deoxygenated cells in the absence of Ca^{2+} , a small and variable fraction of cells became more dense in the presence of quinine. For RBCs deoxygenated with 1.2 mM Ca^{2+} in the medium, however, the fraction of cells that became more dense was greatly reduced when quinine was present. Thus, quinine prevented an increase in cell density only when it was induced by deoxygenation and Ca^{2+} . These results suggest that when reticulocyte-rich SS RBCs are sickled in the presence of Ca^{2+} , K^+ channels are activated and participate in the cell dehydration.

Effects of acidification and Na^+ replacement on the density distribution of SS cells. To test the reticulocyte model's predicted requirement of a high Na^+ influx to maintain the normal volume of reticulocytes, and to see if there were cells with an active $\text{K}:\text{Cl}$ cotransport that could be stimulated by low cell pH, oxygenated, reticulocyte-rich SS cells were incubated either at lowered pH (from 7.4 to 7.0) or in media in which external Na^+ was replaced by the monovalent cation NMG^+ , commonly considered impermeant. The results in Table V and Fig. 5 show that with each of these maneuvers a proportion of cells became more dense. Na^+ replacement had no significant effects on the density distribution of normal RBCs within the same period (not shown).

The presence of 20 mM Na^+ in the media of experiment I in Table V may account for the smaller dehydrating effect of NMG^+ in this experiment than in experiments II and III, in which Na^+ was completely replaced by NMG^+ . In the latter two experiments, in which a substantial fraction of RBCs became denser in glucamine media at pH 7.4, that denser fraction was maximal at 30 min incubation and then decreased. Also, the effects of combining low pH and Na^+ replacement were not additive. Both of these findings could result if the reticulocytes were somewhat permeable to NMG^+ . In Fig. 5, details of the RBC density distribution in one of the experiments reveal that a small fraction of RBCs incubated in glucamine media (at either pH) became persistently very dense, as if these cells were least permeable to NMG^+ . Further study would be needed to resolve these possibilities.

In one experiment SS cells from reticulocyte-rich density fractions were suspended in solution D (pH 7.4), with and without 0.1 mM bumetanide (a known inhibitor of the $\text{Na}:\text{K}:\text{2Cl}$ cotransport), and incubated for 2 h at 37°C. No difference was detected in the density distribution of the cells before and after incubation, with or without bumetanide. This result argues against significant participation of a $\text{Na}:\text{K}:\text{2Cl}$ cotransport in the volume balance of SS reticulocytes.

The proportion of SS cells from each density fraction capable of dehydration by combined acidification and external Na^+ replacement is shown in the experiment of Table VI. After 1 h of incubation in these conditions, roughly similar proportions of cells from each of the three fractions investigated underwent some dehydration, while > 80% of the cells in each fraction behaved in this respect like normal mature RBCs and did not dehydrate. With this blood sample the cells from the upper discocyte fraction that increased in density were considerably enriched in reticulocytes; these could represent the expected cells with simultaneously preserved reticulum and young cell transport properties that have moved down from the lightest density fractions in the process of becoming dense SS RBCs.

To see if the reported low magnesium content of dense SS RBCs (16) could be traced to the fraction of cells that would dehydrate in low Na^+ , low pH media, we measured the total cell magnesium in each cell density fraction. As seen in Table VI, magnesium decreased slightly with increasing cell density, and within each density fraction those cells that shrank after Na^+ replacement and acidification contained less magnesium than those that did not dehydrate.

This experiment demonstrates that many morphologically recognizable SS reticulocytes either lack or have lost the transport properties that mediate dehydration in low Na^+ , low pH media. Conversely, among the cells in each discocyte density fraction exhibiting those transport properties and capable of

mine media (at either pH) became persistently very dense, as if these cells were least permeable to NMG^+ . Further study would be needed to resolve these possibilities.

Table V. Effects of Medium Acidification and Na^+ Replacement on the Density of Reticulocyte-rich SS RBCs

Time min	Percent RBCs attaining density exceeding original values specified											
	Experiment I (density exceeded 1.091)				Experiment II (density exceeded 1.087)				Experiment III (density exceeded 1.091)			
	pH 7.0		pH 7.4		pH 7.0		pH 7.4		pH 7.0		pH 7.4	
	Na	NGM	Na	NGM	Na	NGM	Na	NGM	Na	NGM	Na	NGM
0	0	0	0	0	0	0	0	0	0	0	0	0
15	2.5	2.9	0	0.6	22	15	—	6.0	14	9.6	0	19
30	6.6	6.2	0	0.8	38	25	—	11	19	23	0	22
45	9.2	8.2	0	0.7	39	29	—	5.0	—	—	—	—
60	11	10	0	1.0	42	30	—	5.0	34	36	—	12
120	—	—	—	—	41	33	2.0	7.0	—	—	—	—

SS RBCs with maximum densities of 1.087 or 1.091 and 49–52% reticulocytes were suspended at 2% Hct in either high Na^+ media (Na, solution D) or in glucamine media (NGM) at pH 7.0 or 7.4, and incubated at 37°C. In experiment I the glucamine media differed from the one listed in Methods by containing 125 mM NMG^+ and 20 mM NaCl rather than 145 mM NMG^+ . At the times indicated 0.5-ml aliquots of suspension were taken to examine the RBC density distribution using solutions of Stractan (see Fig. 5 legend). The percent of RBCs whose density exceeded the initial maximum is shown in the table, whereas full details of the RBC density distribution of each timed sample taken in experiment II are shown in Fig. 5.

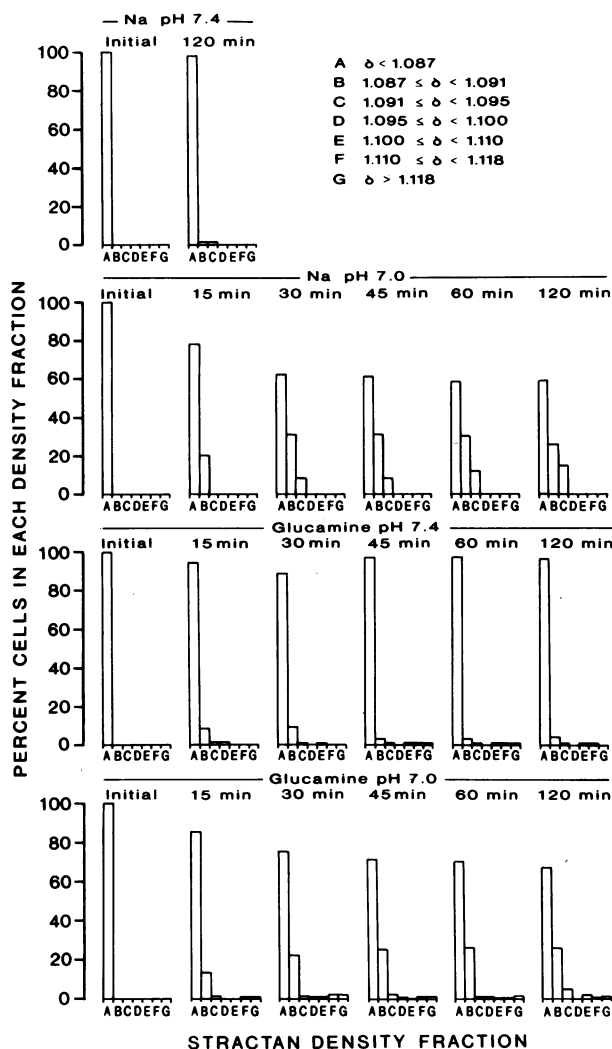


Figure 5. Effects of acidification and Na^+ replacement by NMg^+ on the density distribution of a light, reticulocyte-rich fraction of SS RBCs. An RBC fraction with density $\delta \leq 1.087$, containing 52% reticulocytes, was suspended at 2% Hct in either solution D (with plasma levels of sodium) or "glucamine medium," at pH either 7.4 or 7.0 at 37°C . At the times shown, 0.5-ml aliquots of RBC suspension were layered onto 0.5-ml Stractan solutions of different densities in 1.5-ml plastic tubes. After 2 min centrifugation at 12,000 rpm in an Eppendorf microcentrifuge the Hb content in the pellet was measured and expressed as a percentage of the total Hb in the aliquot. Some of these results are summarized in Table II (as experiment II) for comparison with two similar experiments.

shrinking in this media, there are some that have lost the reticular material.

Distribution of Hb F in SS cell density fractions before and after dehydration by acidification or deoxygenation in the presence of Ca^{2+} . The experiments shown in Table VII were done to examine the possible relevance of the proportion of Hb F in the SS RBCs to their propensity to dehydrate when induced either by activation of the K:Cl cotransport by acidification or by deoxygenation in the presence of Ca^{2+} . It should be kept in mind that in the absence of concomitant hereditary persistence of fetal Hb, the Hb F is virtually all localized in "F cells," and differences in the distribution of Hb F in different cell

fractions reflects the distribution of these high F RBCs and reticulocytes (5, 17).

As first shown by Bertles and Milner (5), the proportion of Hb F was much lower in both the lightest SS cells, highly enriched in reticulocytes, and the dense cell fractions, comprising primarily ISCs, than in the SS discocyte fractions. After 30 min incubation at pH 7.0 the newly dehydrated cells from the reticulocyte-rich fraction showed only a small further reduction in the proportion of Hb F, from 4.5 to 3.3% (mean values of the four experiments in Table VII in which the density of the original light fractions was ≤ 1.087); but the discocyte fractions, in which mean Hb F levels were enriched to 11.4%, yielded dense cell fractions whose mean Hb F levels were markedly reduced, to 5.3%. A pattern similar to these discocyte fractions was seen with the "retic" fraction in the experiment with donor P.H.: this "retic" fraction included denser cells ($\delta \leq 1.091$) than the others, had only 30% reticulocytes, and the initial Hb F of the light fraction was as high as in the discocyte fractions; but the cells that showed an increased density after incubation at pH 7.0 were virtually all reticulocytes, and their mean Hb F level was only 5.3%, similar to the acid-densified cells in the other experiments. Thus, among most of the high F cells enriched in the discocyte fraction, the K:Cl cotransport could not be activated by lowered pH. On the other hand, since there was little depletion of F cells among the light cells undergoing acid-induced dehydration, the presence of a large proportion of Hb F in the cell does not, per se, appear to inhibit acid activation of the K:Cl cotransport. Rather, the high F cells may have a longer survival for other reasons (e.g., inhibition of Hb S polymerization), so that more of them enter the discocyte fraction and have "outgrown" their K:Cl cotransport features. Those cells in the discocyte fraction that dehydrate at lowered pH may thus retain an active acid-stimulated K:Cl cotransport by virtue of their young age, as suggested by the marked enrichment of reticulocytes among the discocytes that increase in density at pH 7.0; their presence in the middle density "discocyte" fraction could reflect their partial dehydration and downward descent towards the dense cell fraction.

As seen in other experiments, deoxygenation resulted in substantial proportions of denser cells only with the original light "retic" fractions, and it was primarily the reticulocytes that increased in density. The marked depletion of Hb F among these newly denser cells is consistent with the partial inhibition of sickling in the high F cells.

Discussion

The hypothesis of a direct reticulocyte origin of ISCs and other dense SS RBCs was based partly on the predictions of the reticulocyte model developed in the previous paper. According to that model, the mutual cause-effect relationship between acidification and dehydration in reticulocytes should result in progressive dehydration following either initial slight acidification or dehydration due to net loss of KCl. The present experiments, by demonstrating that SS reticulocytes are susceptible to much more rapid dehydration than the discocytes when deoxygenated in physiological conditions in the presence of Ca^{2+} , establish the feasibility of sickling as an important trigger and part of a continued positive feedback mechanism in this process. In addition, they provide evidence for several predic-

Table VI. Reticulocyte Distribution and Magnesium Content of SS RBC Density Fractions Incubated at pH 7.0 in Glucamine Media

Initial density fractions	Initial values			Final values after incubation					
	Cells in fraction	Reticulocytes	Cell [Mg] ^{T*}	RBCs with density unchanged			RBCs with density increased		
				Cells	Reticulocytes	Cell [Mg] ^{T*}	Cells	Reticulocytes	Cell [Mg] ^{T*}
		%			%			%	
Whole blood	100	15	2.70	—	—	—	—	—	—
Retic	27	38	3.84	83	40	3.91	17	32	3.19
Disc I	23	11	2.59	88	6	2.63	12	25	2.38
Disc II	40	5	2.45	82	4	2.43	18	5	2.25
ISC	10	1	2.12	—	—	—	—	—	—

SS RBCs were separated on discontinuous Stractan gradients into four groups with the following densities: retic, $\delta \leq 1.091$; disc I, $1.091 < \delta \leq 1.095$; disc II, $1.095 < \delta \leq 1.118$; and ISC, $\delta > 1.118$. After incubating the upper three fractions in glucamine media (Na^+ replaced by NMG⁺) at pH 7.0 for 1 h at 37°C, the RBCs whose density exceeded the initial maximum in each group were separated from the rest using the appropriate Stractan solutions. * Total cell magnesium, mmol/340 g Hb.

tions of the model and the hypothesis, such as the need for a high Na^+ influx to maintain a normal reticulocyte volume, and the conservation of young cell transporters among relatively dense reticulocytes, as expected for transitional cells.

An important consideration in the detailed analysis of these results, however, is the impurity and heterogeneity of

reticulocyte preparations obtained by density separation, since this bears on the generality of the conclusions. Apart from the expected age heterogeneity, the data reveal a dissociation between morphological and functional "reticulocytes," i.e., between cells with ion transport properties observed only in young circulating RBCs, and reticulocytes identifiable by the

Table VII. Percent Hb F and Reticulocytes before and after Medium Acidification or Deoxygenation of Density-fractionated SS RBCs

Donor	Density fraction	Initial		After 30 min at pH 7.0					After 30 min deoxygenation				
		Retics	Hb F	Cells with density unchanged		Cells with increased density			Cells with density unchanged		Cells with increased density		
				Cells	Hb F	Cells	Hb F	Retics	Cells	Hb F	Cells	Hb F	Retics
J.E.	Retic	53	4.3	63	3.7	37	3.7	94	83	4.9	17	0	83
	Dics	21	14.9	96	15.7	4	5.2	83	*	*	*	*	*
	ICS	*	3.2	*	*	*	*	*	*	*	*	*	*
K.W.	Retic	64	2.4	28	4.0	72	2.0	95	40	4.4	60	1.4	88
	Disc	2	10.6	95	11.3	5	3.7	91	*	*	*	*	*
	ISC	1	2.8	*	*	*	*	*	*	*	*	*	*
C.E.	Retic	49	5.6	65	6.1	35	3.9	91	*	*	*	*	*
	Dics	12	9.3	96	8.6	4	5.9	99	*	*	*	*	*
	ISC	4	4.3	*	*	*	*	*	*	*	*	*	*
B.W.	Retic	57	5.5	23	3.6	77	3.5	93	57	4.9	43	2.4	86
	Disc	2	8.6	88	10.1	12	3.4	18	*	*	*	*	*
	ISC	1	5.7	*	*	*	*	*	*	*	*	*	*
P.H.	Retic ^l	30	11.3	76	15.5	24	5.3	99	94	13.1	6	5.3	82
	Disc	17	13.4	77	19.5	23	8.2	89	99.9	*	0.1	*	*
	ISC	6	4.3	*	*	*	*	*	*	*	*	*	*

All data (cells, reticulocytes, and Hb F) expressed as percent of the total. Five groups of SS RBCs were isolated on discontinuous Stractan gradients: retic, $\delta \leq 1.087$ (except for donor PH, where the retic fraction marked II included all RBCs with $\delta \leq 1.091$); disc, $1.095 < \delta \leq 1.110$; and ISC, $\delta > 1.118$. After incubating the upper two fractions for 30 min in isotonic high Na^+ buffer (solution D) at 37°C, either oxygenated at pH 7.0 or deoxygenated at pH 7.4, the RBCs whose density exceeded the initial maximum in each group were separated from the rest using the appropriate Stractan solutions. * Not done.

usual supravital staining methods, but without significant activity of young cell transporters. The data suggest that loss of the reticular morphology and the various transporters on maturation proceed at unrelated rates in different subpopulations of young SS cells. In addition, the fraction of reticulocytes responding to the various experimental maneuvers performed in these studies varied in different patients, and sometimes in cells from the same patient on different days, although some patterns of response seemed conserved when the experimental conditions were strictly comparable (see Table I). Because of this heterogeneity and variability, the results, particularly those concerned with experimental tests of reticulocyte model predictions, must be interpreted with less generality and precision than those reported for homogeneous populations of mature RBCs (6, 18).

Test of the reticulocyte models' predictions of a Na⁺ influx mechanism. The observed dehydration of variable fractions of light SS RBCs when incubated in the oxygenated state in glutamine media (at normal pH), as seen in Table V, is consistent with the predicted requirement of a high Na⁺ influx to maintain the normal volume of reticulocytes (1). The pattern of cell dehydration upon incubation at pH 7.0 (Fig. 5 and Table V) suggests that the high Na⁺ influx is not mediated predominantly by a pH_i-modulated Na:H exchange, since this model option predicts cell swelling at pH 7.0 ([1], their Fig. 4 B). However, reticulocytes may vary considerably in their transport properties and it is possible that some of the cells that did not dehydrate swelled at acid pH, due to a Na:H exchange. Further experiments will be needed to assess the heterogeneity of Na⁺ entry paths in reticulocytes.

Effects of deoxygenation on light SS cells: feasibility of the hypotheses on a direct reticulocyte origin of ISCs. Whereas with SS discocytes prolonged alternating oxygenation/deoxygenation protocols were necessary to elicit Ca²⁺-dependent net cation loss and dense cell formation (19–22), a single deoxygenation pulse in normal plasma caused a variable but significant fraction of light cells, mostly with reticular morphology, to become highly permeable to cations and sustain an increase in density within 30 min or less. A fraction of reticulocytes and other light SS cells must therefore be susceptible to rapid dehydration upon deoxygenation in the circulation.

In view of the steep concentration dependence of Hb S polymerization (23) it was surprising that on deoxygenation the light cells, whose Hb concentrations are lowest, should show the largest increases in cation permeability. That the intensity of the permeabilization induced by sickling may not relate directly to the fraction of Hb S that participates in polymer formation, particularly among cells that are dense and dehydrated, was suggested by Mohandas et al. (24). They observed decreased sickling-induced K⁺ fluxes after hypertonic dehydration of SS discocytes and concluded that the spicules resulting from aligned polymers, which formed preferentially at the lower Hb concentrations, were more permeabilizing than the nonaligned polymer masses that tended to form at higher Hb concentrations (25) and which did not produce long spicules. Further work on the comparative morphology of reticulocytes that become dense or stay light after deoxygenation should help test the extent to which spikes relate to permeabilization.

Because of their large permeability changes on deoxygenation, the light SS cell fractions are particularly well suited for

investigating the sickling-induced permeabilizing injury. Although the nature of this injury is still unknown, the present results define some of its main functional properties in light SS cells: (a) When the RBCs are suspended in low Ca²⁺ serum or plasmalike buffers (with excess EGTA), deoxygenation increases the Na⁺ and K⁺ permeability leading to gradient dissipation; Na⁺ gain generally balances K⁺ loss, with little cell volume change. (b) The deoxygenation-induced Na⁺ and K⁺ fluxes are generally much larger in the lighter cell fractions than in the relatively denser discocyte fractions of the same SS blood sample. (c) With the light cell fraction, the presence of normal serum Ca²⁺ has two major effects on those fluxes—a reduction in their absolute size, and an altered balance—so that K⁺ efflux exceeds Na⁺ influx, with net loss of total cation. At least part of this imbalance is attributable to activation of a quinine-inhibitable, Ca²⁺-dependent K flux. (d) As with SS discocytes and AS RBCs (heterozygous for Hb S) (26), light SS RBCs are also permeabilized to Ca²⁺ by deoxygenation. (e) in serum or plasmalike buffer with normal Ca²⁺ levels, deoxygenation induces substantial dehydration within 30 min only among cells in the light fractions; the population of newly formed dense cells is variably enriched in reticulocytes and excludes most of the cells with high levels of Hb F; the densest of these newly dense cells includes some with a morphology resembling ISCs.

These results demonstrate that a fraction of light SS cells, mostly reticulocytes, is susceptible to rapid dehydration after deoxygenation in physiological conditions. This process could lead to the formation of increasingly dense young cells (with or without reticulum), which soon after would become the densest cells (including morphological ISCs) that no longer contain reticulum. Direct ISC formation from a subpopulation of vulnerable reticulocytes therefore represents a viable working hypothesis.

At present we can only speculate about the factors determining the susceptibility of certain light, young SS RBC to rapid dehydration. Observations that the fraction of ISCs or dense cells in each patient is fairly constant in the steady state between crises (27, 28) suggest that there might be similarly constant fractions of reticulocytes susceptible to dehydration. Since polymerization of Hb S is inhibited in high F cells, F reticulocytes should be relatively excluded from the susceptible population if sickling is a necessary step. Other factors that determine steady fractions of ISCs in individual patients might include differences in the numbers of Ca²⁺ pumps, Ca²⁺-sensitive K⁺ or Cl[−] channels (29), or K:Cl cotransporters per cell. It seems less likely that the observed constancy may result from stochastic factors, such as individual variations in the frequency, duration, and intensity of sickling-induced Ca²⁺ permeabilization among young cells.

These experiments also demonstrated two new properties of the sickling-induced cation leak in SS reticulocytes under certain nonphysiological conditions. One is a much larger sickling-related leak of Na⁺ and, to a variable extent, of K⁺ in the absence of external Ca²⁺. Since sickling increases Ca²⁺ permeability in the reticulocytes, the present data do not distinguish between effects of large (physiological) amounts of [Ca²⁺]_o and small increases in [Ca²⁺]_i, in the nanomolar range. Further experiments with SS reticulocytes loaded with Ca chelators should resolve this question. A second new property is the marked increase in the sickling-induced Na⁺ and K⁺ leaks

in SS reticulocytes in the presence of heparin. Investigation of this effect may provide further clues about the mechanism of the permeabilization and will be the subject of separate studies.

The mechanism of rapid dehydration of young SS cells.

One possible mechanism of formation of ISCs and other dense SS cells, derived from the analysis of reticulocyte models (1), was that a fraction of vulnerable reticulocytes, upon deoxygenation and sickling, would increase their $[Ca^{2+}]_i$ levels enough to activate K^+ channels and thus trigger K^+ and Cl^- loss, cell dehydration, and slight acidification (1). Since the K:Cl cotransport is highly pH sensitive in the physiological range, further dehydration and acidification would follow. The experimental results, that rapid dehydration of young cells upon deoxygenation is strictly Ca^{2+} -dependent, demonstrate that the hypothesis is plausible but do not yet prove that the cells dehydrate by that mechanism.

There are two possible triggers for the initial dehydration of SS reticulocytes. One is acidification, which was also shown by Brugnara et al. (2) to dehydrate oxygenated (or carbon monoxide-equilibrated) cells from the light and middle density fractions of SS blood; the other is deoxygenation-induced Ca^{2+} permeabilization. Transient acidification of any cell with a large number of pH_i-sensitive K:Cl cotransporters could result in dehydration, without deoxygenation or sickling. Ca^{2+} -induced dehydration, on the other hand, requires a Ca^{2+} -permeabilizing event such as sickling, since the membrane has a low Ca^{2+} permeability and a large capacity Ca^{2+} extrusion pump (30, 31). Ca^{2+} -dependent dehydration may proceed by two pathways: directly, by activation of K^+ - and Cl^- -selective channels (29, 32), and indirectly, by secondary acidification and activation of K:Cl transporters, as shown by model analysis (1).

The earlier findings of Bertles and Milner, that the proportion of Hb F was low in the dense ISCs (5), could be simply explained if polymerization of deoxy Hb S played an important role in the dehydration process. The results described above confirm a low frequency of high F cells in both the lightest and densest SS cell fractions, and enrichment of F cells in the discocyte fraction. The additional findings, that the F cells in the light fraction are not excluded from dehydration induced by low pH, suggest that Hb F does not inhibit acid-activation of the K:Cl cotransport. This reinforces the likelihood that Hb S polymerization and sickling are important initiating factors in dehydration of the SS reticulocytes. Within the middle density discocyte fraction, the cells that dehydrate at low pH have low F cell proportions similar to the light fraction and are highly enriched in reticulocytes. This is consistent with the possibility that these are mostly low F reticulocytes "on their way down" to becoming ISCs and other dense SS cells. The present findings can account for the reported observation that the fraction of SS discocytes that are volume sensitive is similarly lower in Hb F than the mean value for the discocytes (33).

We analyze next the possible contributions of the two triggering mechanisms. Although Ca^{2+} -independent acidification in the circulation (without deoxygenation) could act as a trigger, the distribution of F cells described above supports the idea that deoxygenation-induced sickling, and not solely acid-stimulated activation of the K:Cl cotransport in the oxygenated SS cells, is important in the early stages of SS cell dehydration. Furthermore, RBCs ordinarily become partially

deoxygenated at the same time as they acidify. Whenever acidification results from local circulatory stasis, deoxygenation and sickling-induced Ca^{2+} permeabilization are also likely. On the other hand, increased Ca^{2+} permeability may occur in the partially deoxygenated venous circulation without additional acidification of the local plasma. The progressive accumulation of total calcium in all SS cell density fractions is positive evidence of past episodes of increased Ca^{2+} permeability, since accumulation is the result of ATP-dependent trapping within endocytic vesicles (34), and increased calcium accumulation must follow from elevated $[Ca^{2+}]_i$. Vesicular calcium accumulation per se does not tell whether the increased $[Ca^{2+}]_i$ was high and prolonged enough to dehydrate the cells by activation of K^+ channels and Cl^- channels. But recent experiments suggest that a rise in $[Ca^{2+}]_i$ from normal values of ~ 10 – 30 to 40 – 50 nM may suffice to trigger dehydration in normal RBCs (35).

Although the present experiments show that external acidification and Na^+ replacement led to dehydration of many cells in the middle, discocyte fraction as well as in the lightest fraction of SS cells, earlier results (36–38), confirmed here, indicate that deoxygenation of the SS discocyte fractions in the presence or absence of Ca^{2+} did not rapidly dehydrate any cells. It follows that those cells within the discocyte fraction that retain the ability to dehydrate by external acidification and Na^+ replacement have lost or lacked the ability of a large fraction of light cells to rapidly dehydrate when deoxygenated in the presence of Ca^{2+} .

Within the constraints of these results, it is possible to outline a modified working hypothesis on the origin of ISCs and other dense SS cells. These would arise primarily from a susceptible young subpopulation of SS RBCs, which excludes the high F cells in which Hb polymerization is inhibited. Repeated partial deoxygenation in the circulation would initiate dehydration of these susceptible cells, generating somewhat denser young cells, many of which may still retain reticular material. The initial dehydration would be Ca^{2+} dependent, resulting from activation of K^+ and Cl^- channels, but not necessarily K:Cl transporters. Among these dehydrated, slightly acidified young cells, those retaining functional K:Cl cotransporters would continue to dehydrate regardless of their state of oxygenation. By the time they attain the high density of ISCs, most would have lost the reticulum and the activity of all young cell transporters. Nonsusceptible reticulocytes and young cells, on the other hand, would mature further to discocytes, exposed to slow Ca^{2+} -dependent dehydration by repeated deoxygenation in the circulation (19, 39). This would generate the observed heterogeneity of cell densities, with a fraction of young cells dehydrating faster than the rest within each density fraction.

Further work will be needed to investigate the transport options represented in SS reticulocytes, to identify the traits that select the cells susceptible to rapid dehydration, and to establish the relative importance of Ca^{2+} -dependent and -independent triggers of young cell dehydration.

Acknowledgments

We wish to thank the Wellcome Trust of Great Britain and the National Institutes of Health (grants HL-28018 and HL-21016) for funds.

References

1. Lew, V. L., C. J. Freeman, O. E. Ortiz, and R. M. Bookchin. 1990. A mathematical model of the volume, pH, and ion content regulation in reticulocytes. Application to the pathophysiology of sickle cell dehydration. *J. Clin. Invest.* 86:100-112.
2. Brugnara, C., H. F. Bunn, and D. C. Tosteson. 1986. Regulation of erythrocyte cation and water content in sickle cell anemia. *Science (Wash. DC)*. 232:388-390.
3. Brugnara, C., and D. C. Tosteson. 1987. Inhibition of K transport by divalent cations in sickle erythrocytes. *Blood*. 70:1810-1815.
4. Brugnara, C., T. V. Ha, and D. C. Tosteson. 1989. Acid pH induces formation of dense cells in sickle erythrocytes. *Blood*. 74:487-495.
5. Bertles, J. F., and P. F. A. Milner. 1968. Irreversibly sickled erythrocytes. A consequence of the heterogeneous distribution of hemoglobin types in sickle cell anemia. *J. Clin. Invest.* 47:1731-1741.
6. Freeman, C. J., R. M. Bookchin, O. E. Ortiz, and V. L. Lew. 1987. K-permeabilized human red cells lose an alkaline, hypertonic fluid containing excess K over diffusible anions. *J. Membr. Biol.* 96:235-241.
7. Lew, V. L., C. J. Freeman, O. E. Ortiz, and R. M. Bookchin. 1988. A new hypothesis on the origin of irreversibly sickled cells: predictions from an integrated reticulocyte model. *Clin. Res.* 36:567A. (Abstr.)
8. Ortiz, O. E., V. L. Lew, and R. M. Bookchin. 1986. Calcium accumulated by sickle cell anemia red cells does not affect their potassium (^{86}Rb) flux components. *Blood*. 67:710-715.
9. Corash, L. M., S. Piomelli, H. C. Chen, C. Seaman, and E. Gross. 1974. Separation of erythrocytes according to age on a simplified density gradient. *J. Lab. Clin. Med.* 84:147-151.
10. Danon, D., and Y. Marikovsky. 1964. Determination of the density distribution of red cell populations. *J. Lab. Clin. Med.* 64:668-674.
11. Lew, V. L., R. Y. Tsien, C. Miner, and R. M. Bookchin. 1982. The physiological (Ca^{2+}) level and pump-leak turnover in intact red cells measured with the use of an incorporated Ca chelator. *Nature (Lond.)*. 298:478-481.
12. Tiffert, T., J. Garcia-Sancho, and V. L. Lew. 1984. Irreversible ATP depletion caused by low concentrations of formaldehyde and calcium-chelator esters in intact human red cells. *Biochim. Biophys. Acta*. 773:143-156.
13. Garcia-Sancho, J. 1985. Pyruvate prevents the ATP depletion caused by formaldehyde or calcium-chelator esters in the human red cell. *Biochim. Biophys. Acta*. 813:148-150.
14. Wiley, J. S., and C. C. Shaller. 1977. Selective loss of calcium permeability on maturation of reticulocytes. *J. Clin. Invest.* 59:1113-1119.
15. Lew, V. L., and H. G. Ferreira. 1978. Calcium transport and the properties of a calcium-activated potassium channel in red cell membranes. *Curr. Top. Membr. Transp.* 10:217-277.
16. Bookchin, R. M., O. E. Ortiz, and V. L. Lew. 1987. Red cell magnesium content and permeability in sickle cell anemia. *Clin. Res.* 35:651A. (Abstr.)
17. Dover, G. J., S. H. Boyer, S. Charache, and K. Heintzelman. 1978. Individual variation in the production and survival of F cells in sickle-cell disease. *N. Engl. J. Med.* 299:1428-1435.
18. Lew, V. L., and R. M. Bookchin. 1986. Volume, pH and-ion content regulation in human red cells: analysis of transient behavior with an integrated model. *J. Membr. Biol.* 92:57-74.
19. Bookchin, R. M., O. E. Ortiz, and V. L. Lew. 1986. Red cell calcium transport and mechanisms of dehydration in sickle cell anemia. In *Approaches to the Therapy of Sickle Cell Anaemia*. Y. Beuzard, S. Charache, and F. Galacteros, editors. INSERM, Paris. 291-299.
20. Bookchin, R. M., O. E. Ortiz, and V. L. Lew. 1987. Activation of calcium-dependent potassium channels in deoxygenated sickled red cells. In *Pathophysiological Aspects of Sickle Cell Vaso-Occlusion*. R. L. Nagel, editor. Alan R. Liss, Inc., New York. 193-200.
21. Ohnishi, S. T. 1983. Inhibition of the in vitro formation of irreversibly sickled cells by cepharanthine. *Br. J. Haematol.* 55:665-671.
22. Ohnishi, S. T., K. Y. Horiuchi, and K. Horiuchi. 1986. The mechanism of in vitro formation of irreversibly sickled cells and modes of action of its inhibitors. *Biochim. Biophys. Acta*. 886:119-129.
23. Hofrichter, J., P. D. Ross, and W. A. Eaton. 1976. Supersaturation in sickle cell hemoglobin solutions. *Proc. Natl. Acad. Sci. USA*. 73:3035-3039.
24. Mohandas, N., M. E. Rossi, and M. R. Clark. 1986. Association between morphological distortion of sickle cells and deoxygenation-induced cation permeability increase. *Blood*. 68:450-454.
25. Asakura, T., and J. Mayberry. 1984. Relationship between morphological characteristics of sickle cells and method of deoxygenation. *J. Lab. Clin. Med.* 104:987-994.
26. Bookchin, R. M., and V. L. Lew. 1981. Effect of a "sickling pulse" on calcium and potassium transport in sickle cell trait red cells. *J. Physiol. (Lond.)*. 312:265-280.
27. Serjeant, G. R., B. E. Serjeant, and P. F. Milner. 1969. The irreversibly sickled cell: a determinant of haemolysis in sickle cell anaemia. *Br. J. Haematol.* 17:527-533.
28. Fabry, M. E., L. Benjamin, C. Lawrence, and R. L. Nagel. 1984. An objective sign in painful crisis in sickle cell anemia. The concomitant reduction of high density red cells. *Blood*. 64:559-563.
29. Ortiz, O. E., R. M. Bookchin, and V. L. Lew. 1988. A new method to measure the distribution of diffusional chloride (Cl) permeabilities in the red cell populations reveals a Ca^{2+} -sensitive Cl permeability in sickle cell anemia reticulocytes. *Clin. Res.* 36:415A. (Abstr.)
30. Ferreira, H. G., and V. L. Lew. 1977. Passive Ca transport and cytoplasmic Ca buffering in intact red cells. In *Membrane Transport in Red Cells*. J. C. Ellory and V. L. Lew, editors. Academic Press, NY. 53-91.
31. Schatzmann, H. J. 1982. The plasma-membrane calcium pump of erythrocytes and other animal cells. In *Membrane Calcium Transport*. E. Carafoli, editor. Academic Press Limited, London. 41-108.
32. Gardos, G. 1958. The function of calcium in the potassium permeability of human erythrocytes. *Biochim. Biophys. Acta*. 30:653-654.
33. Fabry, M. E., J. R. Romero, R. L. Nagel, and M. Canessa. 1989. Sick cells are heterogeneous in volume response: a mechanism for irreversibly sickled cells (ISC) formation. *Clin. Res.* 37:380A. (Abstr.)
34. Lew, V. L., A. Hockaday, M. I. Sepulveda, A. P. Somlyo, A. V. Somlyo, O. E. Ortiz, and R. M. Bookchin. 1985. Compartmentalization of sickle cell calcium in endocytic inside-out vesicles. *Nature (Lond.)*. 315:586-589.
35. Tiffert, T., J. L. Spivak, and V. L. Lew. 1988. Magnitude of calcium influx required to induce dehydration of normal human red cells. *Biochim. Biophys. Acta*. 943:157-165.
36. Tosteson, D. C., E. Carlsen, and E. T. Dunham. 1955. The effects of sickling on ion transport. I. Effect of sickling on potassium transport. *J. Gen. Physiol.* 39:31-53.
37. Tosteson, D. C., E. Shea, and R. C. Darling. 1952. Potassium and sodium of red blood cells in sickle cell anemia. *J. Clin. Invest.* 31:406-411.
38. Bookchin, R. M., and V. L. Lew. 1983. Red cell membrane abnormalities in sickle cell anemia. *Prog. Hematol.* 13:1-23.
39. Gupta, R. K., and F. A. X. Schanne. 1986. ^{19}F NMR measurements of intracellular free calcium in human red cells. *Fed. Proc.* 45:549. (Abstr.)