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Effects of Abnormal Cation Transport on Deformability of Desiccytes

Margaret R. Clark, Narla Mohandas, Vincent Caggiano, and Stephen B. Shohet

Departments of Medicine and Laboratory Medicine and the Division of Hematology (N.M., S.B.S.); the Cancer Research Institute, the University of California (M.R.C., N.M., S.B.S.), San Francisco, California 94143; and the Sacramento Medical Foundation Blood Bank (V.C.), Sacramento, California

We have studied the deformability of subpopulations of red cells from a patient with "desiccytosis," a disorder characterized by increased membrane permeability to potassium and associated with a probable increase in sodium-sodium exchange. Cells become increasingly dehydrated after maturation because of continued potassium loss without compensatory sodium gain, and they exhibit a progressive increase in mean cell hemoglobin concentration (MCHC). This increase in MCHC causes the cells to become undeformable at shear stress values which result in extensive deformation of normal cells. Reduction of MCHC to approximately normal levels by suspending the cells in hypotonic medium restores normal deformability to all but 0.1–0.2% of the cells. These results suggest that the major factor leading to premature destruction in this disorder is whole cell rigidity conferred by increased intracellular hemoglobin concentrations, rather than any associated membrane rigidity.

Key words: red cell, desiccytosis, deformability, MCHC, ektacytometer, Nystatin, dehydration, potassium leak

An important requirement for adequate red cell function is that the cells be able to deform easily in their passage through the circulation. In a number of hemolytic anemias [1] the loss of red cell deformability seems to be a major factor leading to premature hemolysis. One such disorder, originally described by Glader et al [2], is "desiccytosis," or "xerocytosis." Desiccytosis is characterized by a red cell membrane leak for potassium, with attendant water loss and increase of mean cell hemoglobin concentration (MCHC). Two patients who exhibit the characteristic features of this disorder have recently come to our attention, and we have conducted studies using their blood to explore the relationship between whole cell deformability and decreased cell water concentration. Using an ektacytometer to measure deformability of desiccyte subpopulations with progressively increasing MCHC, we have shown that increased MCHC exerts a profound influence upon the deformability of these cells. Artificially dehydrated normal cells exhibited the same dependence upon MCHC for deformability as did the patient cells. Furthermore, reduction

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of the MCHC in the pathologic cells restored their ability to deform, thus identifying hemoglobin concentration as the primary determinant of reduced cell deformability in this disorder.

MATERIALS AND METHODS

Blood was drawn from patients and from normal control subjects into heparinized vacutainers. MCHC were determined using hematocrits measured in a microhematocrit centrifuge and hemoglobin values which were measured using Drabkin's solution containing 0.5% saponin (Hycel, Inc, Houston, Texas). Mean cell volumes were determined from hematocrits and red cell counts were obtained with a Coulter Model A electronic cell counter. Osmotic fragility curves were obtained according to Parpart et al [3].

Lipid extracts were prepared according to Rose and Oklander [4], and lipid phosphorus and cholesterol were determined by the methods of Lowry et al [5] and Zlatkis, Zak, and Boyle [6], respectively. Phospholipid distributions were determined by means of thin-layer chromatography according to Skipski, Peterson, and Barclay [7], with subsequent phosphorus determination by the method of Parker and Peterson [8]. Fatty acid methyl esters were prepared from the lipid extracts by treatment with methanol and sulfuric acid [9], and were analyzed by gas-liquid chromatography by the method of Dodge and Phillips [10].

Membrane proteins were examined by electrophoresis in polyacrylamide gels containing sodium dodecyl sulfate in the manner described by Weber and Osborne [11]. Membranes for electrophoresis were prepared by the method of Dodge, Mitchell, and Hanahan [12], with the exception that 10 mM Tris-HCl (pH 7.4) was used instead of phosphate buffer for hemolysis and washing of the membranes.

For studies involving subpopulations of various densities, blood was centrifuged on Stractan gradients, by the method of Corash et al [13] with minor modifications [14]. Deformability of cells from whole blood and of the separated cells was evaluated using an ektacytometer [15]. Solutions for deformability measurements at various osmolalities contained 10 mM sodium phosphate (pH 7.4) and sufficient NaCl to bring the osmolality to the desired value. Then 40,000-MW dextran was added to a concentration of 25 g/100 ml of buffered saline.

Intracellular concentrations of sodium and potassium were measured with a flame photometer, using samples of cells which had been washed three times in isotonic $MgCl_2$ buffered with 10 mM Tris-HCl to pH 7.4 (Tris-$MgCl_2$). Sodium and rubidium influxes were estimated from the influxes of ^{22}Na and ^{86}Rb from suspensions of cells in phosphate-buffered saline containing glucose and potassium (BSKG),* using standard methods [16]. Passive influxes were measured in the presence of 10^{-4} M ouabain; active fluxes were determined from the increase in fluxes in the absence of ouabain. Ouabain-resistant sodium and potassium effluxes were determined by measuring the release of these ions into Tris-$MgCl_2$ containing 10^{-4} M ouabain during incubation at 37°C. Ion concentrations in the suspending medium were measured by flame photometry after sedimentation of the cells in a Beckman microfuge. First-order rate constants for cation effluxes were obtained from a linear regression analysis of the natural logs of the intracellular ion concentrations as a function of time [17].

*BSKG: 7.808 g NaCl, 0.373 g KCl, 2.302 g $Na_2HPO_4 \cdot 7H_2O$, 0.194 g $NaH_2PO_4 \cdot H_2O$, 2.0 g glucose, made up to 1 liter. Osmolality was adjusted to 290 ± 5 mOsm/kg and pH to 7.4 if necessary.

Artificially dehydrated cells were prepared from the blood of normal control subjects in a manner designed to reproduce both the altered sodium-potassium distribution and the reduced water content of native desiccytes. This was accomplished by incubation of the cells in medium which contained the antibiotic Nystatin (to permit equilibration of sodium and potassium between the external medium and cell interior) and sucrose (to raise the osmolality and dehydrate the cells). Equilibration solutions were prepared to contain sodium and potassium at the concentrations which prevailed in successive native desiccyte subpopulations. For this purpose it was necessary to convert the desiccyte cation concentrations, which were measured in terms of concentration per cell volume, to concentrations in cell water. Because of limited sample volume, direct measurement of subpopulation cell water content was not feasible. Therefore we used the measured MCHC in the various subpopulations to determine the relative water content in each fraction. Then the measured water content and MCHC of unseparated desiccytes was used to estimate the actual water content of the separated cells.

In preparing artificial desiccytes, cell water content was adjusted by preparation of the equilibration solutions with added sucrose to increase the medium osmolality. The osmolality required to produce cells with the desired MCHC was determined empirically in a separate series of experiments. It was found that for normal cells with initial MCHC of approximately 36 g/100 ml, the required osmolality could be predicted on the basis of the relationship: osmolality = 18.05 × MCHC − 334.1 mOsm/kg.

All equilibration solutions contained 10 mM potassium phosphate at pH 7.4. Normal cells were first washed free of plasma in isotonic buffered saline, then were washed once in the appropriate equilibration solution. The cells were resuspended in the same solution to approximately 10% hematocrit, and equilibration of sodium and potassium across the cell membrane was initiated by the addition of Nystatin at a final concentration of 30 μg/ml, using a stock solution of 5 mg Nystatin in 1 ml anhydrous methanol [18]. The osmolality of the medium during the period of increased membrane permeability determined the total ion and water contents per cell; the ion concentrations in the medium determined the final concentrations of sodium and potassium in the water which remained in the cells. The cells were allowed to equilibrate for 30 min at 37°C with gentle agitation. Then the Nystatin was washed out in four changes of equilibration medium without Nystatin. This restored the original permeability barrier to sodium and potassium. Then the cells were washed in isotonic medium, after which MCHC and cation concentrations were determined in the usual manner.

RESULTS

Two patients, a mother and son, with well-compensated hemolytic anemia and no evidence for a hemoglobin abnormality, were referred to us for further study. Hematologic data for these patients are summarized in Table I. These data showed a slight elevation in MCHC in combination with increased proportions of reticulocytes. While the MCHC were not greatly increased in the whole blood, the fact that they were higher than normal in spite of the presence of considerable numbers of reticulocytes, which have significantly low MCHC, suggested the presence of other cells with significantly elevated MCHC. In addition, as illustrated in Figure 1, the osmotic fragility of the cells was reduced in comparison to normal control cells. Measurements of intracellular sodium and potassium, summarized in Table II, revealed a considerable deficit in potassium concentration, without compensatory increase in sodium concentration. The concurrence of increased MCHC, re-

TABLE I. Hematologic Data

	Age	Packed cell volume (%)	Mean cell volume (μ^3)	Mean cell hemoglobin concentration (g/100 ml)	Reticulocytes (percentage)
Mother[a]	38	36.6	104	38.2	8
Son	15	34.9	92	37.6	12
Normal control	37	39.0	91	33.8	0.5

[a]Splenectomized.

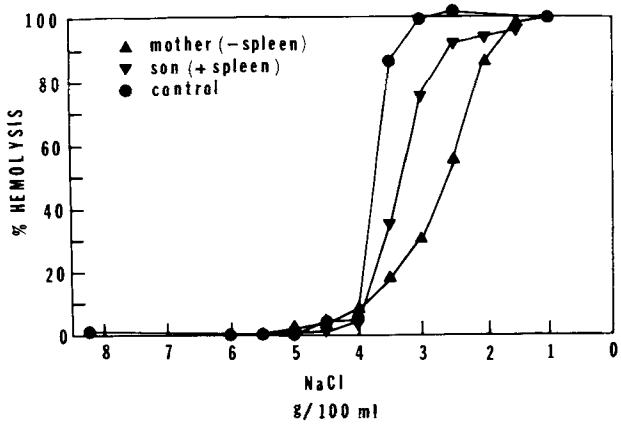

Fig 1. Osmotic fragility curves for patients and normal control.

TABLE II. Red Cell Sodium and Potassium Concentrations

	Na	K	Na + K
Mother	17.0	69.9	86.9
Son	12.8	74.7	87.5
Normal control	7.3	94.7	102.0

Cation concentrations are expressed in units of mEq/liter cells.

duced osmotic fragility, and decreased total monovalent cation concentrations suggested the presence of red cell dehydration secondary to a potassium leak, a phenomenon previously described by Glader et al [2] as "desiccytosis." Wiley et al [19] have described several cases which also appear to be similar to this disorder, but which were termed "stomatocytosis" because of the cell morphology. In fact, the blood from our patients did contain some stomatocytes (Fig 2). The mother's blood also contained some echinocytes and target cells, the latter presumed consequence of splenectomy. However, the essential common feature of our patients and those of the other authors is decreased cell water, rather than common morphology.

Red cell lipids were measured to test the possibility of any abnormality related to the altered cation levels. These data are summarized in Table III. Total lipid phosphorus and cholesterol levels were elevated in the mother, as would be expected in the absence of a spleen [20], but the son's red cell lipid content was essentially normal. The distribution of phospholipid classes revealed a slight increase in the percentage of phosphatidylcholine. These results are similar to those reported by Wiley et al [19]. It also presents a picture not unlike a hemolytic anemia described by Jaffe and Gottfried [21], characterized by an

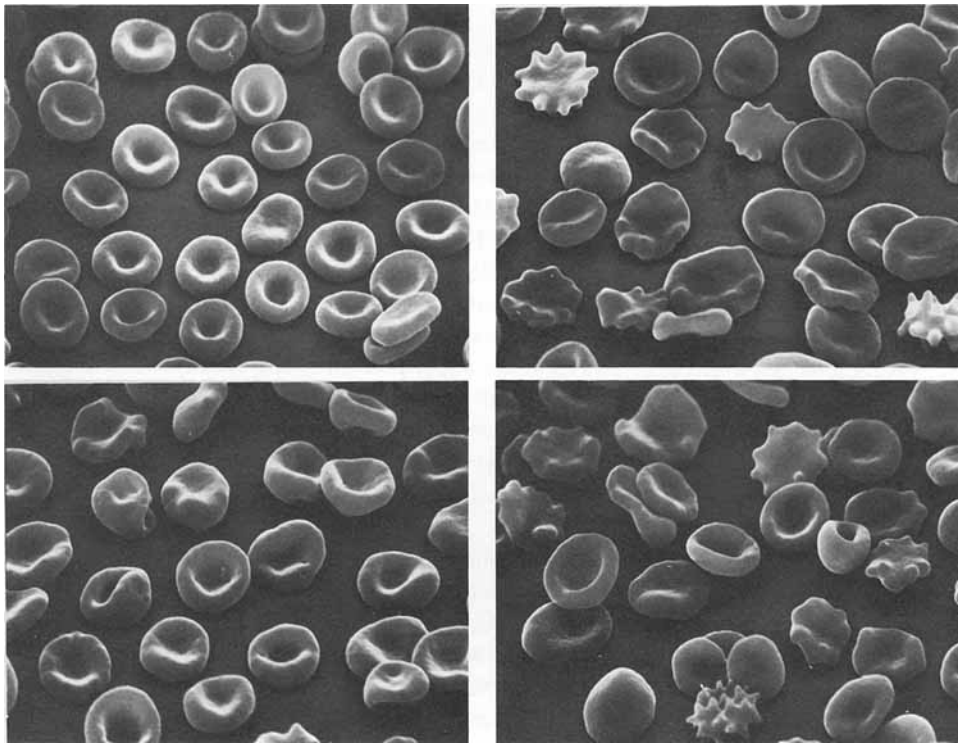

Fig 2. Scanning electron micrographs of patients' cells (original magnification approximately × 2,000): a) Whole blood from son; b) Whole blood from mother; c) Cells from top fraction of mother's cells separated on Stractan gradient; d) Cells from bottom fraction of Stractan gradient.

TABLE III. Red Cell Lipid Data

	Lipid phosphorus ($\mu g/10^8$ cells)	Cholesterol ($\mu g/10^{10}$ cells)	Phospholipid classes (mole %)			
			Sphingomyelin	Phosphatidylcholine	Phosphatidylserine and inositol	Phosphatidylethanolamine
Mother	1.69	1.47	23.8	36.0	15.1	25.0
Son	1.26	1.06	23.9	32.8	15.4	28.0
Normal control[a]	1.22 (0.06)	1.28 (0.07)	26.1 (0.6)	28.7 (1.2)	14.5 (1.1)	30.8 (0.8)

[a]Mean (SD); n = 7 for lipid phosphorus and cholesterol; n = 5 for data on phospholipid classes.

elevation of phosphatidylcholine and associated with reduced turnover of the lipid. It may be relevant that those patients also exhibited reduced potassium and increased sodium concentrations. However, in these cases the cell water was elevated and MCHC was normal [22]. The fatty acid composition of the red cell lipids from our patients was similar to our normal control values (data not shown).

Electrophoresis of membranes on SDS polyacrylamide gels revealed no significant abnormalities in the pattern of major membrane polypeptides.

Because the presence of reticulocytes in hemolytic anemia frequently obscures the properties of mature cells, we separated samples of blood from the mother into subpopulations of progressively increasing density by centrifuging the cells on density gradients of Stractan. One set of experiments employed discontinuous gradients in layers with densities of 1.088, 1.101, 1.110, 1.115, and 1.144 g/ml. A second set of experiments used linear gradients with a density range of 1.079–1.124 g/ml, on a cushion at 1.144 g/ml. Figure 3 summarizes relevant properties of the fractions obtained using the discontinuous gradients. Subpopulations were obtained with progressively increasing hemoglobin concentrations towards the bottom of the gradients. It should be noted that the MCHC in the bottom cell fractions were very high, up to 45 g/100 ml. The most dense normal cells from comparable separations rarely gave MCHC of more than 36 g/100 ml. Desiccyte subpopulations also showed progressive decreases in potassium and total cation concentrations, which is consistent with the picture of progressive dehydration presented by the changes in MCHC.

Reticulocytes were concentrated in the upper portion of the desiccyte fractions, suggesting that cell density increased with cell age. The separated fractions were still heterogeneous in cell volume distribution, although there was a shift toward smaller peak volumes in the more dense cell populations as demonstrated by Figure 4. Cell morphology did not change greatly along the gradient, except that cells in the bottom fraction tended to be more flattened (Fig 2c and d).

We performed measurements of monovalent cation flux components on several desiccyte subpopulations. For influx measurements ^{86}Rb served as an analogue for K uptake. The results are shown in Table IV. Active and passive influx of rubidium was elevated, as was passive efflux of potassium. While the increase in active transport is likely to be a normal response to elevations of intracellular sodium [23], the increase in passive efflux of potassium presumably reflects a primary defect in these cells, which is responsible

Fraction	% total	MCV μ^3	MCHC g/dl	Retics %	Na	K	Na+ K
					meq/l cells		
1	1.4	129	27.0	58	35.3	65.5	100.8
2	22.0	113	36.5	25	19.1	79.2	98.3
3	39.5	99	41.7	2	17.1	69.7	86.8
4	28.7	100	41.2	<1	17.9	61.8	89.7
5	8.5	91	45.3	∽0	19.5	51.1	70.6

Fig 3. Properties of desiccytes separated on Stractan gradients. MCV, mean cell volume.

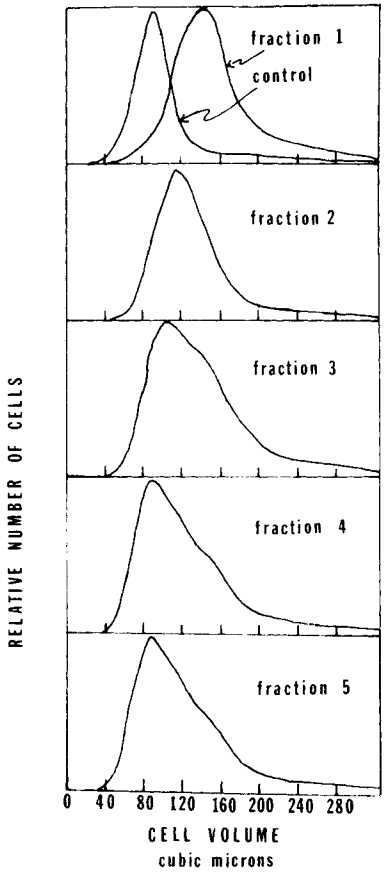

Fig 4. Volume distributions of desiccyte subpopulations from gradient separation.

for the net loss of potassium and water. The presence of this potassium leak provides further evidence for the identification of the desiccytosis syndrome in these patients.

Unilateral uptake of ^{22}Na isotope was greatly increased; most of this uptake probably represents increased sodium-sodium exchange, since the intracellular sodium concentrations are only moderately elevated. This interpretation is supported by our results on passive efflux of potassium. Measured passive K efflux was markedly less than the isotopic ^{22}Na uptake. Had the isotopic measurements represented a mass influx of Na, one would have expected abnormally high intracellular cations and increased water content. In fact, the actual intracellular cation and water concentrations indicate a potassium loss in excess of sodium gain, with overall decrease in cation and water content. Glader's patients also showed high sodium-sodium exchange [24]. The passive sodium efflux in separated desiccytes and various fractions was not markedly abnormal.

Deformability measurements using the ektacytometer showed that the whole blood of both patients contained a double population of cells, one deformable, one undeformable. Figure 5 shows the diffraction pattern obtained with the ektacytometer from the mother's cells, in comparison with that for normally deforming cells. The circular pattern superimposed upon the center of the ellipse indicates the presence of cells which do not deform

TABLE IV. Sodium and Potassium Flux Components in Desiccytes

	^{86}Rb influx[a]		K efflux rate constant (h^{-1})	^{22}Na influx (mEq/liter cells/h)	^{23}Na efflux rate constant (h^{-1})
	Passive	Active			
Unseparated cells	–	–	0.035	–	0.069
Separated fractions					
1	–	–	0.038	–	0.062
2	0.202	0.744	0.028	14.2	0.074
3	–	–	0.036	–	0.086
4	–	–	–	–	–
5	0.372	0.516	0.052	12.2	0.108
Normal control	0.070	0.274	0.014	1.2–2.4	0.066

Rb influx was determined from the measured uptake of ^{86}Rb isotope from phosphate-buffered saline containing 5 mM K; influx in the presence of 10^{-4}M ouabain was considered to represent passive flux; active flux was taken to be the increment in ^{86}Rb uptake in ouabain-free medium. ^{22}Na influx was measured analogously, using the ^{22}Na isotope. K and Na effluxes were determined by measuring the rate of appearance of the ions in the initially Na- and K-free incubation medium (see Materials and Methods).

[a] ^{86}Rb/ml cells / ^{86}Rb/ml medium × h^{-1}.

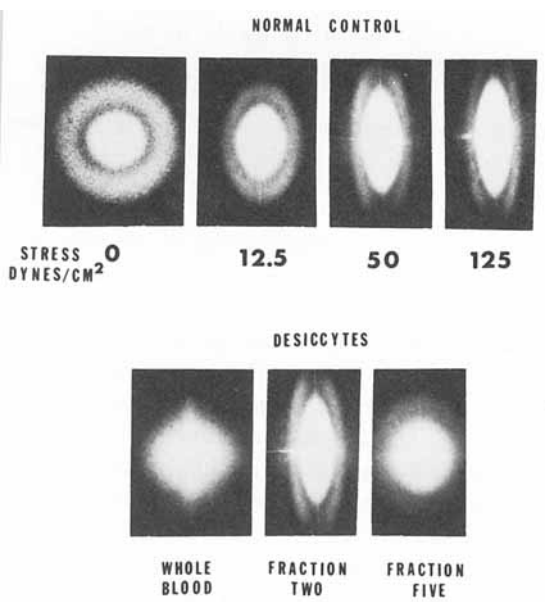

Fig 5. Ektacytometer diffraction patterns: a) Diffraction patterns of normal cells subjected to increasing shear stress (elliptical patterns indicate cell deformation); b) Diffraction patterns of desiccytes at 125 dynes/cm^2 shear stress. Note superimposition of elliptical and circular patterns for whole blood, indicating the presence of both deforming and nondeforming cells. Gradient centrifugation separated blood into deformable and undeformable subpopulations, as indicated by diffraction patterns from fractions two and five.

under the shear stress imposed by the instrument. Centrifugation of the blood on the Stractan gradients separated the deformable from the undeformable cells. Fractions one and two deformed normally, fractions three, four, and five were completely undeformable in the isotonic medium used. Figure 5 illustrates diffraction patterns of fractions two and five.

The association between lack of deformability and increased MCHC suggested the possibility that the failure of these cells to deform was simply the result of their high hemoglobin concentrations. To test this hypothesis, we prepared dextran-containing solutions of progressively lower osmolalities, with the idea that hypotonic swelling of the desiccytes might restore cell deformability. As summarized in Table V, when suspended in sufficiently hypotonic medium, virtually all of the cells became deformable. Improved separation of the more dense cells using continuous density gradients allowed us to estimate the residue of undeformable cells at 100 mOsm to be approximately 0.1%–0.2% of the total cell population. In all cases, suspensions of the cells in hypotonic medium were spun down in a microhematocrit centrifuge to eliminate the possibility that we were observing the deformation of partially hemolyzed cells under these conditions. In no instance was hemolysis observed.

To confirm the hypothesis that MCHC played a major role in limiting the deformability of dehydrated cells, we prepared artificially dehydrated cells to mimic the desiccyte subpopulations in cation concentrations and MCHC. This was done as described in Methods and Materials, using the antibiotic Nystatin to modify the sodium and potassium concentrations of normal cells. Table VI summarizes the cation and hemoglobin concentrations of artificial desiccytes for comparison with corresponding properties of the native desiccyte subpopulations described in Figure 3. Table VI also shows the ion concentrations and osmolalities which were employed to produce the desired alterations.

The artificially dehydrated cells showed the same kind of dependence of deformability upon medium osmolality as the patient cells. Table VII lists the MCHC obtained for native and artificial desiccytes in the medium in which they became completely deformable. There is some indication that lower osmolalities may have been required to restore deformability to the native cells than to the artificially dehydrated ones. This may reflect a greater range of MCHC among the patient cells, with some high MCHC cells remaining undeformable under conditions at which the majority of the cells deform normally.

TABLE V. Deformability of Desiccytes in Media of Various Osmolalities

	Medium osmolality				
Fraction	285	225	180	150	100
1	+				
2	+				
3	–	+			
4	–	–	+		
5	–	–	–	+[a]	+[b]

Symbols: –) undeformable; +) deformable.
[a] 5% of fraction 5 still undeformable.
[b] 1–2% of fraction 5 still undeformable.

TABLE VI. Preparations of Artificial Desiccytes

	Buffer cations (mEq/liter)		Buffer osmolality	Intracellular cations (meq/liter)		MCHC
	Na	K	(mOsm/kg)	Na	K	(g/100 ml)
Nystatin-treated normal cells						
Simulated desiccyte fraction						
2	30.1	124.2	292	30.6	94.5	34
3	34.5	139.3	364	21.5	61.2	36
4	29.5	105.1	372	21.7	79.3	40
5	32.6	85.6	505	23.6	56.7	46
Control	11.7	153.3	284	8.2	102.6	35
Untreated cells	160.3	5.4	284	7.9	101.6	37

Normal cells were treated with Nystatin in appropriate buffers to simulate the cation concentrations and MCHC of the native desiccyte subpopulations. The various "fractions" and control were incubated in the indicated buffers with 30 μg/ml of Nystatin. The buffer osmolalities were adjusted to the tabulated values with sucrose. Untreated cells were kept in phosphate-buffered saline throughout.

TABLE VII. Hemoglobin Concentrations at Which Artificial and Native Desiccytes Deform

	MCHC at 285 mOsm (g/100ml)	Osmolality at which cells deform (mOsm/kg)	MCHC at permissive osmolality (g/100 ml)
Artificial desiccytes	34	285	34
	36	285	36
	40	225	35
	46	150	32
Patient desiccytes	36	285	36
	39	180	31
	40	150	31
	45	100	33

Normal cells were artificially dehydrated by equilibration in buffers containing Nystatin to adjust ion and water concentrations to values close to those in native desiccyte subpopulations. In the first column the resulting MCHC in isotonic medium are given in comparison to the native desiccyte subpopulations. Deformability measurements were conducted in media of successively lower osmolalities, to determine the osmolality which would permit the cells to deform. These "permissive" osmolalities are tabulated in the second column. Corresponding MCHC at these osmolalities are listed in the final column.

DISCUSSION

As has been discussed by LaCelle [1], deformability in red cells is sensitive to alterations in three distinct cellular properties: the ratio of membrane surface area to cell volume, the flexibility of the membrane, and the viscosity of the cell interior. There are various pathologic conditions in which one or another of these factors appears to limit the

ability of the red cells to deform and, presumably, to limit the cell lifetime in the circulation. A decrease in surface to volume ratio with formation of more spherical cells can occur through loss of cell membrane or increase of volume with uptake of water. The first mechanism may be important in autoimmune hemolytic disease [25], the latter in certain cases of stomatocytosis [26]. Increased rigidity of the cell membrane may promote red cell removal by the spleen and other reticuloendothelial organs when ATP depletion allows the intracellular accumulation of calcium, as in stored blood [27]. Finally, the studies here suggest that increased internal viscosity of cells with a high MCHC, without a reduction in surface-to-volume ratio or increased membrane rigidity, may contribute to red cell destruction. Such a mechanism has been postulated for splenic entrapment of hereditary spherocytes [28], but because those cells also may have experienced both membrane loss and increase in membrane stiffness, the contribution of their high MCHC is not readily isolated.

Although the membrane appears not to have a direct influence upon lack of deformability of desiccytes, the disorder is the result of abnormal membrane function. The particular molecular defect is unknown; its primary manifestations include increased potassium permeability, a probable acceleration of sodium-sodium exchange, and possible increase in the proportion of phosphatidylcholine with decrease phosphatidylethanolamine. Whatever the fundamental abnormality, its effect on MCHC through its influence on cation permeability would appear to have a profound effect upon cell deformability and provide a possible mechanism for premature red cell destruction.

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