Reversible sodium pump defect and swelling in the diabetic rat erythrocyte: Effects on filterability and implications for microangiopathy

(aldose reductase/inositol/glycated albumin/ATPase/protein kinase C)

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ABSTRACT We have found a defect in the ouabain-sensitive Na⁺,K⁺-ATPase (Na⁺ pump, EC 3.6.1.37) of erythrocytes from streptozotocin diabetic rats. This defect was accompanied by an increase in cell volume and osmotic fragility and a decrease in the cytosolic K⁺/Na⁺ ratio. There was also a doubling in the time needed for diabetic erythrocytes to pass through 4.7-μm channels in a polycarbonate filter. Our data are consistent with a primary defect in the erythrocyte Na⁺ pump and secondary changes in cell volume, osmotic fragility, K⁺/Na⁺ ratio, and cell filterability. All were reversed or prevented in vivo by insulin or the aldose reductase inhibitor Sorbinil. Protein kinase C agonists (phorbol ester and diacylglycerol) and agonist precursor (myo-inositol) reversed the Na⁺ pump lesion, suggesting that protein kinase C-dependent phosphorylation of the 100-kDa subunit regulates Na⁺ pump activity and that insulin can influence erythrocyte protein kinase C activity. Ouabain inhibition of the erythrocyte Na⁺ pump also produced increases in cell size and reductions in rates of filtration. Theoretical treatment of the volume changes also predicts reduction in filterability as a consequence of cell swelling. We suggest that enlarged erythrocytes could play a role in the evolution of the microvascular changes of diabetes mellitus.

A decrease in ouabain-sensitive Na⁺,K⁺-ATPase (Na⁺ pump, EC 3.6.1.37) activity has been found in diabetic lens, nerve, and glomerulus (1-3). This Na⁺ pump impairment was corrected by aldose reductase inhibitors or by normalization of blood sugar with insulin. In contrast, Na⁺ pump activity in diabetic erythrocytes (RBCs) is a subject of controversy, with reports of increases (4, 5) and decreases (6, 7). We therefore initiated a study of the Na⁺ pump in erythrocytes of rats rendered diabetic with streptozotocin. The streptozotocin diabetic rat provided a reliable source of RBCs whose time-integrated exposure to hyperglycemia was determined by measurement of glycated albumin. Since nondiabetic RBCs must deform considerably to pass through small capillaries, we were especially interested in learning whether such a putative Na⁺ pump lesion would influence the volume and hence filterability of affected RBCs.

MATERIALS AND METHODS

Diabetic Rats. Male Sprague-Dawley rats (175 g) were injected with streptozotocin (50 mg/kg, i.p.). Diabetic rats had hemoglobin A₁C (HbA₁C) levels between 9% and 16%, glycated plasma albumin levels between 2.5% and 5%, and blood glucose values between 250 and 850 mg/dl. This range of hyperglycemic values permitted correlation of the severity of diabetes with the degree of impairment of Na⁺ pump activity. The aldose reductase inhibitor Sorbinil (d-6-fluoro-spiro (chro-

man-4,4'-imidazoline)-2',5'-dione; a gift from Pfizer Diagnostics) was given by gavage (20 mg/kg per day). Diabetic rats were made euglycemic by daily s.c. injection of lente insulin (2 international units, Eli Lilly).

Glycated Proteins. Glycated albumin and HbA₁C were separated from their unmodified forms with Glycogel B (Pierce) and quantitated as before (8). This method does not underestimate the extent of glycation if the Glycogel B column is not overloaded. Recovery of proteins was >97%, and glycated and unmodified proteins ran true initially and upon rechromatography.

RBC Ghost Preparation. Ghosts were made by the method of Reinila et al. (9) from heparinized ventricular blood (obtained 3 min after i.p. injection of Nembutal). This method produced unsealed sheets of RBC membrane. Protein was measured according to Bradford (10).

Na⁺ Pump Activity. Na⁺ pump ATPase activity was measured in RBC membrane fragments by the method of Reinila et al. (9). [γ-32P]ATP (1000-3000 Ci/mmol; 1 Ci = 37 GBq) was purchased from New England Nuclear. Nucleotides were separated from 32P with Norit (11). ATP concentrations much above 1 mM could not be used, since we found a 37% inhibition of activity at 3 mM ATP and virtually complete inhibition at 7.5 mM ATP when the concentration of free Mg²⁺ was 5 mM. Such substrate inhibition has been previously reported in the Na⁺ transport system (9). In the case of ATP, V_max was determined from data in the linear portion of the Lineweaver-Burk plot, prior to the onset of substrate inhibition. Na⁺ pump activity in intact cells was measured as 86Rb transport, according to DeLuise et al. (12). 86RbCl (1-35 Ci/g, New England Nuclear) was extracted from RBCs with 5% (wt/vol) trichloroacetic acid after uptake by the Na⁺ pump.

Measurement of RBC Na⁺, K⁺, ATP, and Sorbitol Content. Na⁺ and K⁺ measurements were made with a Perkin-Elmer atomic absorption spectrometer. RBCs were washed with isotonic MgCl₂ prior to lysis in hypotonic (5 mM) LiNO₃ (13). ATP was measured with luciferase (14), and sorbitol with sorbitol dehydrogenase (15); values were expressed as mean ± standard error (coefficient of variation < 3%).

RBC Osmotic Fragility. Osmotic fragility was measured in RBCs washed three times with isotonic NaCl (pH 7.4) containing 10 mM Tris, 10 mM glucose, 5 mM each CaCl₂, KH₂PO₄, and MgSO₄, and 0.25% bovine serum albumin. Cells were incubated for 30 min at 37°C at a 5% hematocrit in the above solution, adjusted to the indicated osmolarities with NaCl. After centrifugation, released Hb was measured at 412 nm.

Correction of Na⁺ Pump Impairment by Protein Kinase C Agonists and Insulin. Diabetic RBCs were incubated in glucose (10 mM)-enriched isotonic medium (pH 7.4 and 37°C) with different agonists or precursors at the concentrations and for the time periods indicated (Table 2). Na⁺ pump

Abbreviation: RBC, erythrocyte.
activity was measured with $^{86}$Rb as described above. The protein kinase C inhibitor 1-(5-isoquinolinesulfonyl)-2-methylpyrrolidizine (H7) was purchased from Sigma.

**RBC Volume Measurements.** Packed cell volume was measured by adding $[^3]$H]inulin (1 μl of 1 μCi/ml) to 250 μl of heparinized blood and determining the number of RBCs per ml in a separate aliquot. A cell pellet was formed (200 × g, 3 min, 4°C) in a capillary tube (1 × 75 mm). Pellet volumes were corrected for trapped plasma with $[^3]$H]inulin, and packed cell volume was calculated on the basis of cell number. Coulter (electrical) volumes were measured as described (16).

**RBC Filterability.** Cells were washed three times and theuffy coat was discarded. Cells were diluted with phosphate-buffered isotonic saline containing 1% bovine serum albumin (filtered), adjusted to a hematocrit of 15–20%, and counted. Filtration times were proportional to hematocrits between 10% and 30%. RBC filterability was expressed as the time necessary for 2 ml of sample (3 × 10$^8$ cells) to pass through a 4.7-μm Nuclepore filter under suction (5 cm of water; 1 cm of water = 98.06 Pa). The scintiled-glass filter support added 1 sec to filtration times for nondiabetic as well as diabetic RBCs. This time was subtracted from total filtration time, as were the times for buffer filtration. Filters were soaked in phosphate-buffered saline/albumin overnight and measurements were made at 37°C.

**RESULTS**

**ATP and Sorbitol Measurements.** Rat RBC ATP levels were 2.26 ± 0.09 mM (n = 3), as reported by Lehninger (17). In diabetic RBCs, ATP levels were 1.64 ± 0.03 mM (n = 3). Reduced levels of ATP were not found in RBCs of diabetic rats treated with insulin for 10 days or in RBCs of insulin-treated diabetic humans (18). Cytoplasmic sorbitol levels were 10 μM in nondiabetic RBCs and 50–60 μM in diabetic RBCs.

**Activity of the Ouabain-Sensitive Na$^+$,K$^+$-ATPase.** Na$^+$ pump activity in RBC membrane fragments from untreated diabetic rats was reduced 35–50%. The size of the Na$^+$ pump defect correlated with the severity of diabetes (r = 0.83; n = 14) determined by levels of (nonenzymatically) glycated plasma albumin (Fig. 1, ①). The normal ouabain-insensitive ATPase activity (6.2 nmol of P$_i$ released per min per mg of membrane protein) was not diminished in RBCs of diabetic rats. We also measured Na$^+$ pump activity as $^{86}$Rb uptake by intact RBCs in the presence of diabetic or normal plasma or isotonic buffers. Values in nondiabetic (240 nmol of Rb per hr per 10$^8$ cells) and diabetic (138 nmol Rb per hr per 10$^8$ cells) RBCs showed differences (>41%) comparable to those measured in RBC ghost fragments and were not influenced by plasma.

**Effects of Insulin and Sorbinil on the Development of the Na$^+$,K$^+$-ATPase Defect.** Na$^+$ pump impairment (measured in RBC ghosts) reached a maximum by day 10 after the onset of streptozotocin diabetes (Fig. 2, ①). Insulin treatment prevented Na$^+$ pump impairment when begun at the time of streptozotocin injection and reversed the established defect after 8 days. The Na$^+$ pump defect was prevented when Sorbinil was given from the time of streptozotocin injection.

**Kinetic Parameters for the Na$^+$ Pump and Cytoplasmic Na$^+$ and K$^+$ Measurements.** We measured $K_m$ for ATP, Na$^+$, and K$^+$ in nondiabetic and diabetic RBCs (Table 1). While there were minimal changes in the $K_m$ for Na$^+$ and K$^+$, the $K_m$ for ATP in diabetic cells was doubled. The $V_{max}$ was unaltered for the diabetic Na$^+$ pump. The mol ratios of cytosolic K$^+$ to Na$^+$ were 19.2 ± 2.6 (n = 8) for nondiabetic RBCs and 15.1 ± 3.4 (n = 8) for diabetic RBCs (P < 0.02). The corresponding packed cell volumes were 64.0 ± 2.8 μm$^3$ for nondiabetic cells and 79.6 ± 2.7 μm$^3$ for diabetic cells. These data indicate Na$^+$ pump impairment in diabetic RBCs and suggest that the observed water accumulation is secondary to increases in cytoplasmic Na$^+$$. There was an inverse correlation between the K$^+$/Na$^+$ ratio and RBC volume (r = −0.55, n = 16, P < 0.05).

**Osmotic Fragility.** Fifty percent of diabetic RBCs underwent lysis at 165 mosmol/kg (Fig. 3). RBCs from nondiabetic rats exhibited a comparable degree of lysis at 120 mosmol/kg. At 165 mosmol/kg, the extent of hemolysis was 3-fold greater in diabetic cells. The time course for development of increased osmotic fragility resembled that for development of the Na$^+$ pump defect (Fig. 2, ①). The degree of increased osmotic fragility was related to the magnitude of Na$^+$ pump impairment.

![FIG. 1.](image1) **FIG. 1.** Relationship between plasma glucoalbumin levels and RBC volumes and Na$^+$ pump activity. Na$^+$ pump activity was measured as $^{86}$Rb influx, in nondiabetic and diabetic RBCs. We found a negative correlation (r = −0.83, n = 14, P < 0.01) when the Na$^+$ transport data were plotted against percent glycation of plasma albumin. Packed cell volumes were measured for nondiabetic and diabetic RBCs and plotted (as a percentage of nondiabetic RBC volume) against the corresponding glycated plasma albumin values. We found a positive correlation between these parameters (r = 0.96, n = 8, P < 0.005).

![FIG. 2.](image2) **FIG. 2.** Time course for the development and reversal of the Na$^+$ pump lesion and osmotic fragility. Na$^+$ pump ATPase activity was measured daily in RBC ghost membrane fragments, following a single injection of streptozotocin. We show one of three time courses (①), all of which were in good agreement. We observed a 40% decrease in Na$^+$ pump activity by 10 days after streptozotocin administration. When Sorbinil treatment was initiated 10 days following streptozotocin injection, the decline in Na$^+$ pump activity was largely reversed after 10 days of treatment (point indicated by arrow). Osmotic fragility was also reversed by Sorbinil treatment. After a single injection of streptozotocin, RBC osmotic fragility was measured daily at 150 mosmol/kg (①). Results are given as the mean values for data obtained for three rats.
impairment (Fig. 2). Prevention of Na\(^+\) pump impairment by Sorbinil treatment also prevented increased osmotic fragility.

**Volume Changes.** Na\(^+\) pump impairment in diabetic RBCs was associated with an 11–29% increase in packed cell volume (Fig. 1, ○) and Coulter volume. Measurements of Coulter volume exhibited a bimodal distribution, with both peaks shifted toward larger volumes in diabetic cells. The bimodal distribution in Coulter volume may reflect differences in the orientation of RBCs as they pass the Coulter electrode. The magnitude of the shift (>20%) agreed with the packed cell volume increases. Increases in packed cell volume were related to the severity of diabetes (Fig. 1). Comparable swelling was observed whether packed cell volume measurements were made in plasma or isotonic Tris-HCl (pH 7.4).

**Passage of Nondiabetic and Diabetic RBCs Through a Nucleopore Filter.** We compared the times required for movement of nondiabetic and swollen cells through the 4.7-μm channels of a polycarbonate Nucleopore filter. A swelling of 22% in the RBCs of streptozotocin diabetic rats resulted in a doubling of the time required for the filtration of 3 x 10⁶ cells. The degree of cell swelling correlated with the prolongation of filtration times (Fig. 4, r = 0.96, n = 19).

**Mimicking Effects of Diabetes on RBC Na\(^+\) Pump Activity, Cell Volume, Frailty, and Filterability with Ouabain.** We used ouabain to inhibit the Na\(^+\) pump and to compare changes in volume to those in filterability. At 500 μM, ouabain produced virtually complete inhibition of Na\(^+\) pump activity. At 100 μM, ouabain caused 50% inhibition, which is often encountered in the chemically diabetic rat. The induction of a Na\(^+\) pump defect with ouabain also produced increases in osmotic fragility (data not shown). The correlation between cell swelling and reduced filterability was the same whether Na\(^+\) pump impairment was caused by hyperglycemia or ouabain (Fig. 4).

We did not correlate Na\(^+\) pump impairment in the ouabain-exposed RBCs with the degree of swelling because osmocytic inhibition occurs rapidly (<1 min), whereas cell swelling evolves slowly over a period of hours owing to slow rates of Na\(^+\) influx (19).

**FIG. 3.** Osmotic fragility. Diabetic (○) and nondiabetic (□) RBCs were exposed to the indicated osmolalities and hemolysis was quantitated on the basis of released hemoglobin.

**FIG. 4.** Correlation of cell volume and filterability. Cell volume was measured as packed cell volume. Filterability was expressed as the time taken for 3 x 10⁶ cells to pass through the 4.7-μm channels at —5 cm of water. Data are from diabetic RBCs (○) from nondiabetic RBCs not treated with ouabain (□) or from nondiabetic RBCs incubated for 90 min at 37°C with 100 μM (△) or 500 μM (□) ouabain.

**Table 2.** Effect of various agonists on Na\(^+\) pump activity

<table>
<thead>
<tr>
<th>Agonist or precursor</th>
<th>Time, min</th>
<th>Na(^+) pump activity, % control</th>
<th>Inhibition of stimulation by H7 (20 μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diacylglycerol* (200 μM)</td>
<td>60</td>
<td>89</td>
<td>Yes</td>
</tr>
<tr>
<td>Phorbol 12-myristate</td>
<td>13-acetate (10 μM)</td>
<td>60</td>
<td>90</td>
</tr>
<tr>
<td>myo-Inositol (2 mM)</td>
<td>120</td>
<td>95</td>
<td>Yes</td>
</tr>
<tr>
<td>Insulin (1 μM)</td>
<td>60</td>
<td>95</td>
<td>Yes</td>
</tr>
</tbody>
</table>

Diabetic RBCs were treated with the above agents at 37°C for the time periods shown. Na\(^+\) pump activity was determined in intact RBCs by ⁴²Rb uptake. Values are representative of three different experiments done in duplicate. The uncorrected Na\(^+\) pump activity in diabetic RBCs was 63% of that found in normal RBCs (236 nmol of Rb per hr per 10⁹ cells).

*1-Oleoyl-2-acetyl-sn-glycerol.

**DISCUSSION**

Our data suggest that impairment of RBC Na\(^+\) pump activity and the accompanying cell swelling merit attention as puta-
tive contributors to the pathogenesis of diabetic microangiopathy. We observed in vivo restoration (in <10 days) of Na⁺ pump activity by normalization of blood glucose and/or inhibition of aldose reductase. Protein kinase C agonists restored Na⁺ pump activity in vitro. The Na⁺ pump subunits of diabetic cells appeared normal in number and have the potential for normal function, providing that hyperglycemia and/or its biochemical consequences are reversed. The 8-day time course for Na⁺ pump correction by daily insulin injections (given in the morning) is not a limiting value. A 32% defect in the RBC Na⁺ pump of human type I diabetics was corrected by 24 hr of continuous insulin infusion (24). We found reversal of the effect by insulin in vitro after 60 min (Table 2).

We have documented a doubling in the $K_m$ for ATP of the Na⁺ pump in diabetic RBCs, and this could account for a decrease in Na⁺ pump activity of about 33%. We have measured the $K_m$ for ATP three times in RBCs from diabetic animals with impaired Na⁺ pump activity, and in every case the doubling was observed. The changes in $K_m$ offer a possible explanation (in combination with changes in α-subunit phosphorylation) for the observed changes in Na⁺ pump activity. The effect may be exacerbated by the observed decrease in ATP concentration (~28%), which is in the range that can be explained, in part, by the observed increases in cell volume. The reduced ATP concentration is perhaps paradoxical in view of the significantly (30–50%) diminished Na⁺/K⁺ transport activity, which is normally responsible for about one-quarter of RBC ATP consumption (25). Here we do not exclude the possibility that other metabolic features of the diabetic RBC can reduce ATP concentrations.

We have presented biochemical data that implicate protein kinase C and provide a context for understanding Na⁺ pump impairment in the diabetic RBC and its correction in vitro by some agonists. It is noteworthy that correction of the Na⁺ pump with insulin is prevented by a selective inhibitor of protein kinase C (21). These data, however, do not provide a link between the polyl pathway and the impaired Na⁺ pump.

In our diabetic animal model, hyperglycemia reaches maximal levels between days 3 and 4 following streptozotocin injection (8). Thus, hyperglycemia is well established for more than 5 days before maximal Na⁺ pump impairment is observed. On the other hand, once established, the level of Na⁺ pump impairment correlates well with levels of glycated plasma albumin (Fig. 1). These findings do not exclude the important possibility that insulinoprivia (as distinct from hyperglycemia) contributes to the observed Na⁺ pump impairment. Moreover, it has been observed that insulin can activate some phospholipases and thereby increase levels of diacylglycerol in fibroblasts and myocytes (26, 27). This effect of insulin may result from a GTP-binding-protein-dependent activation of phospholipase C (26) and is in harmony with the data that implicate protein kinase C in the regulation of the Na⁺ pump (Table 2).

Modest levels of sorbitol are found in diabetic RBCs (50–100 μM) in contrast to the levels (8–30 mM) found in other diabetic tissues, such as lens, glomerulus, and nerve (1–3). Thus, sorbitol-associated increases in cell volume cannot account for the observed increases in volume. More likely the increase in volume is due to the decreased rate of Na⁺ extrusion. The observation that this Na⁺ pump impairment is prevented as well as reversed by Sorbinil suggests one or more loci of convergence between the polyl pathway and regulation of the Na⁺ pump (28, 29).

We have documented three immediate adverse consequences of Na⁺ pump impairment: an increase in RBC volume and a consequent increase in osmotic fragility and decrease in filterability. In correlation with our ouabain experiments, all of these changes were reversed when the Na⁺ pump was normalized with Sorbinil or insulin. Because of Na⁺ pump impairment, already swollen diabetic RBCs are less able to contend with further swelling, since they are closer to the bursting point. This is in keeping with the concept that membrane area is a limiting parameter in the capacity of RBCs to adapt to changes in shape and volume (30).

The data in Fig. 4 show that a 22% increase in cell volume produces a doubling in the filtration time for RBCs. In attempting to understand these data, three connected theoretical questions occur. (i) What can account for the very large amplification factor between cell volume and filterability? (ii) Is it reasonable to expect similar amplification of volume changes in vivo? (iii) Could changes in RBC filterability be responsible for some of the pathological consequences of diabetes?

We find it useful here to introduce an objective measure of the capacity of an individual RBC to pass through a small channel. Such a measure is provided by the diameter of the smallest channel through which a given cell can pass without rupture and without causing blockage. We call this measure the "critical diameter." The force of friction between an RBC and the capillary wall must approach infinity as the critical diameter of the cell approaches the capillary diameter. Further, since it requires only one cell to plug a channel, the flow of a heterogeneous suspension of cells will be dominated by those members that offer the greatest resistance. Thus, if other factors are held constant, the flow rate of suspension of cells through a channel should be approximately proportional to the difference between the maximum critical diameter of the cells and the capillary diameter.

Micromechanical studies have demonstrated that the RBC membrane is very cable-like in behavior and that the surface area decreases. Phospholipases A and G and other enzymes that increase the fluidity of the RBC membrane can account for changes in RBC volume observed in vivo (30). If an RBC of given area and volume is passed through a series of progressively smaller capillaries, there will be a point at which the cell no longer has the reserved membrane area required to enclose the (long, thin) shape it must adopt while squeezing through the passage.

In the Appendix, we present a geometrical derivation that allows one to compute the RBC critical diameter given its surface area and volume. We find that the critical diameter of a normal rat RBC (2.47 μm) is surprisingly close to the diameter of the smallest capillaries (~3.5 μm). We also show that, to a good approximation, the critical diameter increases in proportion to the ratio of cell volume over surface area.

Thus, in accord with our in vitro observations, there is a simple mechanism whereby small amounts of cell swelling are amplified into large changes in filterability.

The critical diameter will increase whenever volume increases or surface area decreases. This in fact is the case in older RBCs, where losses of membrane area in excess of 10% are not uncommon and associated decreases in Na⁺ pump activity may cause concomitant increases in volume. Further, since the channel diameters of our filtration assay are larger than those of the smallest rat capillaries, there is every reason to expect that the effects of cell swelling in vivo will be even larger than what we observe in vitro. We conclude that the cell swelling we have documented is a likely cause of pathology. More experiments to directly test this hypothesis and to precisely delimit the nature, rate of onset, and clinical course of the consequences of RBC swelling would be useful.

Of all the chronic complications of diabetes mellitus, the microangiopathy appears most likely to be influenced by RBC swelling and the resulting reduction in microperfusion. Microangiopathy cannot be attributed solely to RBC swelling, however, since extrinsic factors such as plasma viscosity, known to increase in consequence of the increases in fibrinogen concentration (31), as well as accelerated base-
ment membrane thickening (32) and possibly analogous changes in other formed elements including polymorphonuclear leukocytes and platelets, may also contribute to reduced flow. Nevertheless, our data indicate that swollen diabetic RBCs may participate in the evolution of diabetic microangiopathy.

**APPENDIX**

Consider an RBC of given surface area, $A$, and volume, $V$, confined in a channel with diameter equal to the critical diameter, $D_c$. By definition of the critical diameter (see text), the channel must be so narrow as to cause an infinitesimal violation of the constraints of constant surface area and volume. This means that at the critical diameter the configuration of the cell will be such that the cell volume, $V$, is the largest volume that can possibly be enclosed by the area, $A$, in a way consistent with the constraints imposed by the channel. In this configuration, the surface of the cell will conform to that of the confining channel over a certain "contact length," $L_c$. At either end of this cylindrical segment, hemispherical caps will close the cell surface. In other words, at the critical diameter, the cell is forced to take on a "cigar" geometry. The surface area of the cigar geometry is

$$A = \pi D_c^2 + \pi D_c L_c,$$

whereas the volume of the cigar is

$$V = \frac{1}{6} \pi D_c^3 + \frac{1}{4} \pi L_c D_c^2.$$  

We now solve Eq. 1 for $L_c$ and substitute into Eq. 2. After rearrangements and cancellations we obtain a cubic equation for $D_c$:

$$\frac{\pi}{12} D_c^3 - \frac{1}{4} AD_c + V = 0.$$  

In the rat, $V$ has an average value of 66 $\mu$m$^3$ and the average value $A$ is $\approx 109$ $\mu$m$^2$. The corresponding values in the human are 87 $\mu$m$^3$ and 143 $\mu$m$^2$ (33). In either case the nondimensional ratio $V^2/A^3 << 1$. We are therefore able to explicitly solve Eq. 3 in terms of a power-series expansion;

$$D_c = (4V/A)\left(1 + (\pi 16/3)V^2/A^3 + \ldots \right).$$

From this expression we calculate that $D_c$ for the rat RBC is $\approx 2.59$ $\mu$m (vs. 3.5 $\mu$m for the diameter of the smallest capillaries). As a second example, consider what happens if one reduces the surface area by 10% and simultaneously increases cell volume by 10%. In this case, Eq. 4 predicts a critical diameter of 3.27 $\mu$m, which is perilously close to the diameter of the smallest capillaries. Eq. 4 also demonstrates that to first order, the expansion is proportional to the ratio of volume to surface $(V/A)$.

The analysis of the critical diameter presented here has incorporated only the most irreducible physical and geometrical constraints of constant volume and surface area. For example, we have neglected the effects of bending and shear elasticity of the membrane. The actual RBC will fail to satisfy this ideal limit, and thus the experimental $D_c$ will be even larger than predicted by Eq. 4.

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