Reduced Erythrocyte Deformability in Diabetes

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SUMMARY

The flow properties of individual erythrocytes have been studied in glass micropipets 4 μ in internal diameter. The pressure gradient required to establish a standard oscillatory movement over a 130-μ path in a three-second time period was measured in paired studies comparing diabetic and control erythrocytes suspended in Ringer solution. The pressure requirement was regularly elevated for the diabetic erythrocytes, averaging 50 per cent greater than the controls. Studies of erythrocytes comparing alloxan-diabetic rats with control rats demonstrated a similar elevation in required pressure. Red cells from subjects with hereditary spherocytosis offered less flow resistance than diabetic cells, and red cells from rheumatoid arthritics required no pressure increment. When erythrocytes are ejected from a 4-μ micropipet they return quickly to a discoid shape using stored elastic energy. Slow motion photography revealed that diabetic erythrocytes restore their shape less rapidly than nondiabetic erythrocytes, indicating that their reduced deformability is due to an elevation of either intraerythrocyte or membrane viscosity rather than to increased resistance to bending. Diabetes is regularly associated with an increased intracellular hemoglobin AIc; it is possible that hemoglobin AIc could raise intraerythrocyte viscosity. The observed disturbance in flow properties of individual erythrocytes is subtle. It would affect the flow of blood, particularly through active muscle, and modify the pressure exerted by individual erythrocytes on the muscle capillary wall.

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An erythrocyte can pass through vessels smaller than its own diameter because of its ability to alter its discoid shape. Normally 8 μ in diameter, it is capable of passing through glass tubes whose diameter is only 3 μ.[1] Following passage, the red cell returns quickly to its discoid shape. This erythrocyte property is usually referred to as deformability. Reduced red cell deformability has previously been demonstrated to occur in thalassemia, sickle cell disease, and spherocytosis.[2] Ditzel examined the filterability of diabetic erythrocytes using Millipore filters and found it to be impaired immediately after diabetic ketoacidosis.[3] He attributed this to dehydration of erythrocytes in ketoacidosis. The Millipore filters he used have a nonuniform pore size; improved technology has now produced polycarbonate filters of much more uniform pore size. Schmid-Schönbein and Volger, using 5-μ polycarbonate filters, measured the flow of 10 per cent suspensions of diabetic erythrocytes in their own plasma. They found that an increase in pressure gradient was required for the passage of diabetic erythrocytes, and they concluded that diabetic erythrocytes had an impaired deformability compared with normal red cells.[4] Barnes et al. used 5-μ polycarbonate filters at constant pressure and found an impediment in flow only in red cells from diabetics with complications.[5] In both studies, aggregation-promoting properties of diabetic plasma could have accounted for the observed differences. Dintenfass has reported increased "rigidity" of diabetic red cells measured by an indirect means.[6] Because reduced erythrocyte deformability in diabetes was not considered unequivocally established, a direct study of the flow properties of red cells suspended in Ringer solution has been carried out. An

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unexpectedly uniform degree of flow impairment in capillary-sized micropipets has been demonstrated.

METHODS

Fasting venous blood from nonhospitalized, ambulatory, subject volunteers was drawn into vacuum tubes containing dipotassium ethylenediamine tetraacetate. The nine diabetics studied all had established overt diabetes: five were being managed with insulin, three with oral sulfonylurea agents, and one by diet only. Duration of diabetes varied from three to 40 years. The six rheumatoid arthritics were chosen because of their chronic, symptomatic disorder. Five had classic rheumatoid factor–positive disease; their Westergren sedimentation rates varied from 24 to 96 mm. per hour. One had chronic arthritic symptoms without rheumatoid factor and a normal sedimentation rate. Their plasma protein changes were known to be similar to those found in diabetes.[7] Six individuals with hereditary spherocytosis were studied, three before splenectomy; two of these were studied more than six weeks after splenectomy as well and the data averaged: four were members of one family and two of another. Control subjects were selected from laboratory personnel and ambulatory, afebrile, outpatient volunteers known to have a recent, normal, fasting plasma glucose value. In all but the first four human diabetes studies, paired control subjects were of the same sex and matched within eight years for age.

Studies were also performed on age-matched pairs of Sprague-Dawley rats weighing about 200 gm. Diabetes was induced by intraperitoneal injection of 100 mg. per kilogram of alloxan monohydrate freshly dissolved in citrate-saline buffer (pH 4.0).

Red cells were isolated by centrifugation at 1,500 g for 20 minutes. The plasma and buffy coat were removed and the erythrocytes were washed once in physiologic saline and once in albumin-containing Ringer solution. They were then resuspended, diluted a hundredfold, in the Ringer solution. The Ringer solution contained sodium 140 mEq. per liter, calcium 3.5 mEq. per liter, potassium 4.5 mEq. per liter, bicarbonate 1.9 mEq. per liter, 2-amino-2-hydroxymethyl-1,3-propanediol (Tris) 40 mM per liter, and glucose 5.5 mM per liter. It was brought to pH 7.4 with 1 M hydrochloric acid. Bovine plasma albumin (Pentex), 0.25 per cent, was added to halt echinosis formation.

Glass micropipets were made from 5¾-inch disposable pipets (Scientific Products) drawn by hand over a small Bunsen burner. The tip area was drawn to an inner diameter of at least 4 and less than 5 μ measured with a previously calibrated ocular micrometer. A nearly parallel, wall segment of at least a 200-μ length was required (figure 1). To avoid fluid movement due to capillarity, the pipets were filled with Ringer solution before use. One drop of the erythrocyte suspension was placed on a slide below an elevated, plastic cover slip. The tip of the micropipet was maneuvered under the slip into the drop by a micromanipulator. To make measurements, a single red cell was aspirated into the micropipet. A reservoir apparatus, patterned after that of Weed and LaCelle[8] and equipped with a water manometer, was used to measure the pressure difference during erythrocyte motion (figure 2). The folded cell was moved back and forth at a constant rate by pulling and pushing the syringe plunger. Early and again later in the study, a movie camera was mounted on top of the microscope, and motion pictures (18 frames per second) were taken to examine erythrocyte conformational dynamics and the regularity of cyclic motion within the pipet. Two single frames from one of the films are shown (figure 1). The films showed that substantial erythrocyte shape change occurred with direction reversal.

When the microscopist had established satisfactorily stable oscillatory motion, the pressure change sufficient to cause a red cell to traverse 130 μ and back every three seconds was recorded by a second observer. Measurements on seven different erythrocytes from each subject were taken. Only paired studies successfully carried out in the same pipet were utilized. Each micropipet could be used for only one or two paired studies. The pipets differed slightly in size and taper from each other.

In the last four diabetic studies, the classification of the red cells (diabetic versus control) was not known to the experimenters. Statistical analyses were performed using a Tektronix system and programs written by one of us (DEM) based on standard formulas: F- and t-distribution probabilities are calculated to the nearest 0.0001 by the system using a Tektronix program.

RESULTS

Five male and four female diabetics (age range, 12 to 89 years) were compared with four male and five female control subjects (age range, 14 to 67 years). The diabetics' fasting plasma glucose levels ranged from 150 to 258 mg. per deciliter (mean, 209 mg. per deciliter). The pressure gradients observed are shown in table 1. In all nine paired studies, the

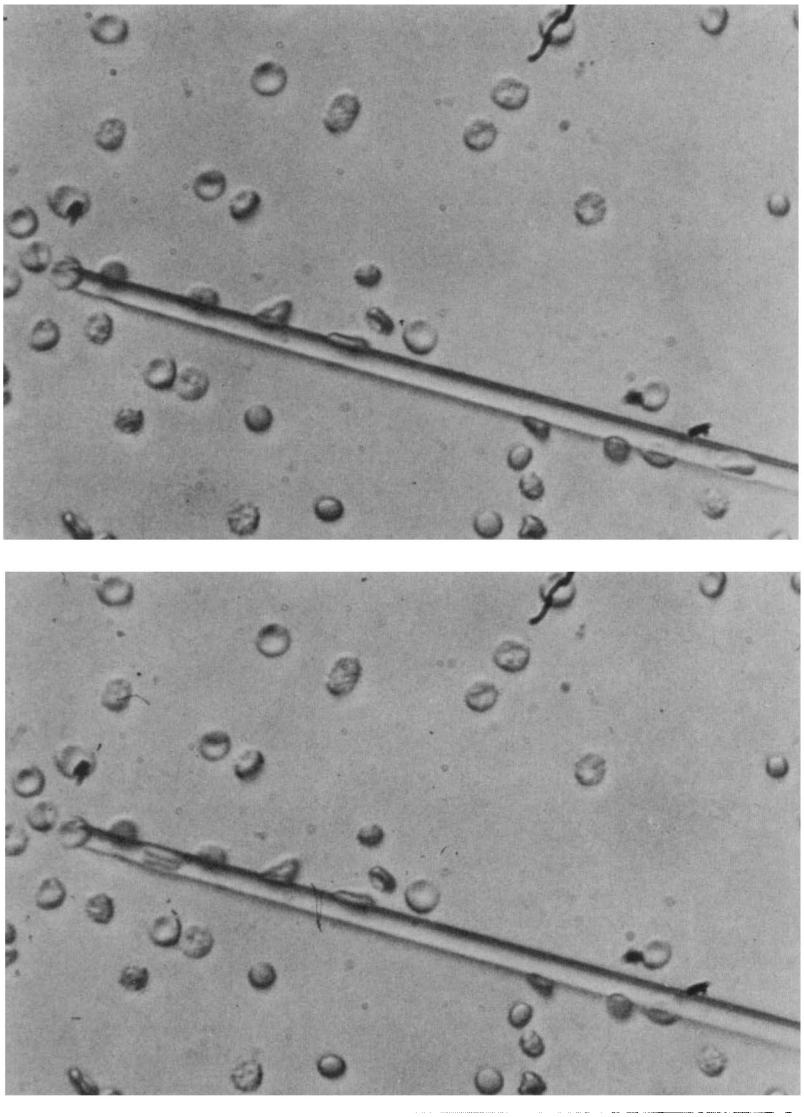


FIGURE 1

The shape and range of movement during pressure measurement of an erythrocyte in a 4-μ pipet are shown. The two photographs are from a 16-mm. color film taken at 18 frames per second. At the top, the erythrocyte is at one end of its movement path; in the bottom figure, it is at the other end (26 frames, or 1.45 seconds, later). The red cell is elongated to 10 μ and rolled on itself so that it looks like a thick crepe suzette or taco shell.

diabetic value exceeded the normal value. Statistical evaluation, carried out using the paired t test, demonstrated that the difference in pressure was highly significant. A diabetic-to-control ratio is also shown. The ratio reduces the scatter produced by pipet differences. Diabetic age, duration, and degree of fasting glucose elevation were not significantly correlated with the ratio of diabetic-to-control pressure in this small study ($r = 0.44, -0.61, +0.31$, respectively).

Each value in table 1 is the mean of pressure gradients of seven cells from each subject. The within-subject standard deviation varied from 0.5 to 2.1 mm. H_2O (mean, 1.2 mm. H_2O) for the diabetic cells and 0.3 to 1.9 mm. H_2O (mean, 0.9 mm. H_2O) for control cells. The replicate standard deviation was proportional to the mean pressure; the observed coefficient of variation of replication averaged 16 per cent. The standard error of each pressure shown in table 1, therefore, averaged 0.4 mm. H_2O and never exceeded 0.8 mm. H_2O.

The observations on human diabetic erythrocytes were supplemented by studies of experimental animals. Diabetic Sprague-Dawley rats were paired with healthy control rats. The diabetic animals' blood glucose concentration ranged from 256 to 490 mg. per deciliter (mean, 297 mg. per deciliter). In all five studies, the diabetic rat had an elevated pressure gradient compared with its nondiabetic control. On average, both human and rat diabetic red cells required half again as much pressure for the standard oscillatory

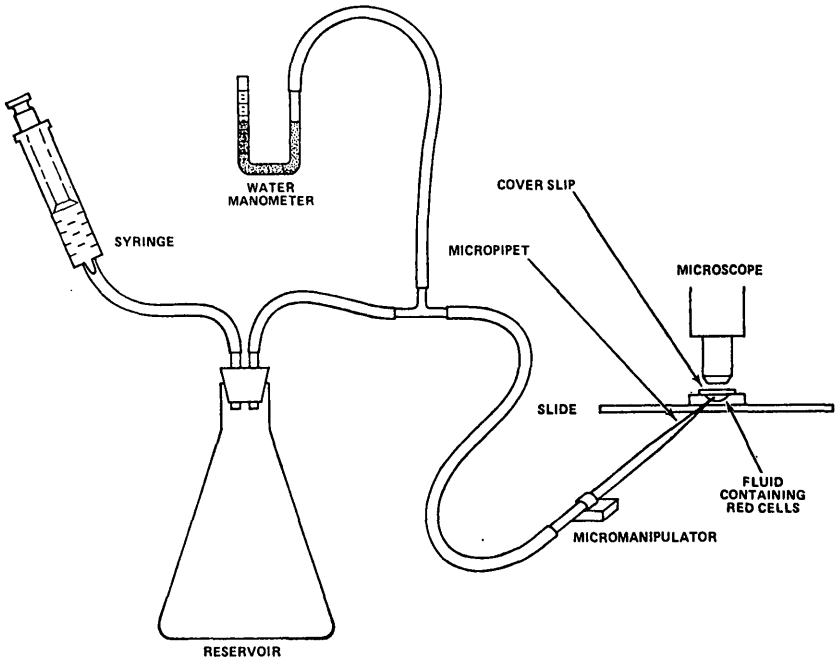


FIGURE 2

Diagram of the system used to measure red cell deformability. The Erlenmeyer flask used has a volume of 500 ml. The water manometer was filled with water blackened with India ink to make reading easier. The microscope used was a Leitz with a triocular eyepiece for photography.

motion as did nondiabetic cells.

The effect of an artificially high glucose level in the suspending medium on nondiabetic, human, erythrocyte, flow properties was examined by exposing them to Tris Ringer solution containing 400 mg. per deciliter (24 mM per liter) glucose for one hour before study. Nondiabetic cells showed no increase in required pressure compared with cells from the same subject kept in 5 mM per liter glucose–Tris Ringer solution.

Erythrocytes from patients with two other chronic disorders were tested. Erythrocytes from six subjects with hereditary spherocytosis aged four to 45 years were examined in paired studies because of the known reduction in deformability associated with this disorder. The spherocytic red cells required a slightly higher pressure gradient than did control cells (table 2). The increase is not as striking as that found in diabetes. Their mean corpuscular hemoglobin concentration (Coulter) was 36.2 ± 0.7 gm. per deciliter (mean ± S.D.) compared with 34.6 ± 0.9 gm. per deciliter for 63 healthy subjects. In six other paired studies, rheumatoid arthritic red cells were found to have the same pressure gradient requirement as red cells from healthy subjects (table 2).

We investigated the possibility that cell-size changes produced by blood processing might be different for diabetic and normal erythrocytes. A model-S Coulter Counter was used to determine the mean corpuscular volume (MCV) of normal and diabetic erythrocytes in their original plasma and then suspended in the Tris Ringer solution after processing. In four paired experiments, diabetic MCV increased from 85 to 92 μ^3 and control MCV from 86 to

TABLE 1

Pressure differences required for standard erythrocyte flow pattern in 4-μ micropipets
Diabetes Studies

Experiment	Pipet	Human Erythrocytes Control	Diabetic	Ratio of D/C
		Pressure (mm. H_2O)		
1	A	2.86	4.07	1.42
2	A	4.64	6.00	1.29
3	B	5.57	10.14	1.82
4	C	3.57	4.71	1.32
5	D	10.30	17.10	1.66
6	E	8.40	11.70	1.39
7	F	4.70	8.20	1.74
8	G	4.64	7.36	1.59
9	H	3.75	4.93	1.31
		5.38 ± 0.81	8.25 ± 1.40	1.50 ± 0.07
		Group Means ± S.E.		Ratio Mean ± S.E.
		Paired t test: t = 4.00, P = 0.004		

Experiment	Pipet	Rat Erythrocytes Control	Diabetic	Ratio of D/C
		Pressure (mm. H_2O)		
1	I	6.29	10.17	1.62
2	J	3.29	3.86	1.17
3	J	3.43	6.57	1.92
4	K	5.14	7.71	1.50
5	K	7.33	10.29	1.40
		5.10 ± 0.79	7.72 ± 1.20	1.52 ± 0.12
		Group Means ± S.E.		Ratio Mean ± S.E.
		Paired t test: t = 4.72, P = 0.009		

TABLE 2

Experiment	Pipet	Control	Spherocytic	Ratio of S/C
		Spherocytosis Studies		
1	L, N*	4.07	5.07	1.25
2	M, N*	4.43	5.29	1.19
3	O	8.7	11.0	1.26
4	P	4.0	4.0	1.00
5	Q	6.00	6.14	1.02
6	Q	6.14	6.72	1.09
		5.56 ± 0.74	6.37 ± 1.00	1.14 ± 0.05
		Group Means ± S.E.		Ratio Mean ± S.E.
		Paired *t* test: t = 2.41, P (one-tailed) = 0.03		
		Rheumatoid Arthritis Studies		
Experiment	Pipet	Control	Rheumatoid	Ratio of R/C
1	R	6.29	5.14	0.82
2	S	11.29	12.71	1.13
3	S	10.29	9.29	0.90
4	T	3.29	3.00	0.91
5	T	3.14	3.86	1.23
6	U	10.86	10.57	0.97
		7.53 ± 1.55	7.43 ± 1.62	0.99 ± 0.06
		Group Means ± S.E.		Ratio Mean ± S.E.
		Paired *t* test: t = 0.24, P = 0.82		

*Studies were performed before and after splenectomy and the pressures averaged.

98 μ^3. The slight rise in mean corpuscular volume appears to be an artifact of cell size determination in the Coulter Counter, since the hematocrit of both normal and diabetic erythrocytes suspended in the Tris Ringer solution was actually slightly lower than when suspended in their native plasma.

India ink particles, 0.5 to 1.0 μ in diameter, were used to determine the pressure required to cause the same rate of cyclic flow for an erythrocyte-free Ringer solution. The pressure difference required to move the particles at the same velocity as erythrocytes was less than 0.5 mm. of water.

To determine the effect of the presence of more than one cell in the pipet on flow resistance, two erythrocytes from a healthy subject were aspirated into a micropipet so that they were within 20 μ of each other. Pressure differences required to produce the standard velocity movement were 20 to 30 per cent higher than for a single erythrocyte. In an obviously tapered pipet, almost no increase in gradient pressure was necessary, suggesting that even minimal tapering of the pipet causes one cell to offer considerably more flow resistance than the other. Change in red cell diameter during transit within the pipets used in the study could also be seen in the motion pictures.

The ability of erythrocytes to return to a discoid shape was studied by ejecting diabetic and normal red cells from 4-μ pipets and photographing their rate of unfolding at 64 frames per second. Diabetic erythrocytes averaged 24 frames (0.38 sec.) to unfold most of the way to their normal discocyte shape, while normal red blood cells averaged 16 frames (0.25 sec.) to unfold at least as completely.

DISCUSSION

Studies in 4-μ pipets have revealed a regular impairment of erythrocyte deformability in diabetes. Considering the observed limit of measurement reproducibility, the regularity of the finding in diabetes is quite striking. Red cells of rheumatoid arthritics failed to show a similar increase, even though rheumatoid arthritis is associated with plasma protein changes similar to those in diabetes. The increased pressure requirement in diabetes was greater than in hereditary spherocytosis, a disorder known to reduce red cell deformability. Previous studies of spherocytic cells utilized pipets 3 μ in diameter. This size micropipet causes a normally discoid red cell to be reshaped into a cylinder with hemispheres at both ends. In this circumstance cells whose surface-to-volume ratio is reduced, such as spherocytic erythrocytes, show a marked resistance to flow. In 4-μ pipets, spherocytic cells have much less flow difficulty, probably because conversion to a cylindric shape is no longer required.

No relationship between decreased red cell deformability and degree of hyperglycemia, age, or duration of diabetes was detected. It should be noted that paired studies are necessary. Even with a far more extensive study, a real effect of hyperglycemia or duration of diabetes could be missed because of the limitations of this technique. The basis for the impaired deformability observed must be determined so that a more reliable method of assessment can be used in further studies.

Hochmuth has shown that, when red cells flow in small tubes, a fluid layer exists between the outer red cell membrane and the wall of the tube:[10] it is in this layer that fluid movement (shearing) occurs. The thickness of the fluid layer is controlled by the pressure being exerted on it by the red cell membrane. An increase in this pressure narrows the layer and raises resistance to flow. The less elastic (more rigid) the membrane the narrower would be the gap between the red cell membrane and the tube wall, raising resistance to flow. The possibility that reduced red cell membrane elasticity is the basis for decreased deformability in diabetes was tested by ejecting red cells from pipets. If increased rigidity of red cells had been present, the diabetic erythrocytes would restore themselves to a discoid shape more rapidly than healthy

erythrocytes, much as a stiffer spring snaps open more rapidly when it is released. The observed slowing of return of diabetic red cells to their discoid shape indicates that an entirely different mechanism is operating. Motion pictures of red cell movement in the 4-μ pipet indicated that slight tapering was present. Studies introducing two red cells at the same time showed that the pressure increase was less than expected, further supporting the existence of tapering. During each oscillation, the red cell's shape changes slightly, being compressed when moving toward the tip and expanded when moving away (figure 3). This change is resisted both by the elastic properties of the red cell membrane and by the cell's viscous resistance. The photographic observation of slowed return to discoid shape of diabetic red cells is best explained by an enhanced viscosity, since the alternative explanation—reduced rigidity—would be associated with decreased resistance to flow in the pipet. During the red cell's migration, if the cell's viscosity is elevated, the fluid layer between it and the wall will be narrowed as it moves toward the pipet tip, raising the pressure required for its motion. Increased red cell viscosity in diabetes will produce both increased flow resistance and slowed return to discoid shape.

Two cells elements determine red cell viscosity. The inner environment of an erythrocyte is a Newtonian hemogoblin solution whose viscosity is severalfold that of plasma.[11] Increased intracellular viscosity would decrease red cell deformability. Elevation of intracellular hemoglobin concentration in hereditary spherocytosis may contribute to the observed increased flow resistance. Red cell hemoglobin concentration is normal in diabetes, but diabetic erythrocytes have a specific increase in hemoglobin AIc (Hb AIc) content. Hb AIc is produced when glucose becomes attached to normal hemoglobin.[12] Hemoglobin AIc could increase the intracellular viscosity of the diabetic erythrocyte if the attachment of glucose to hemoglobin increases its interaction with adjacent hemoglobin molecules.

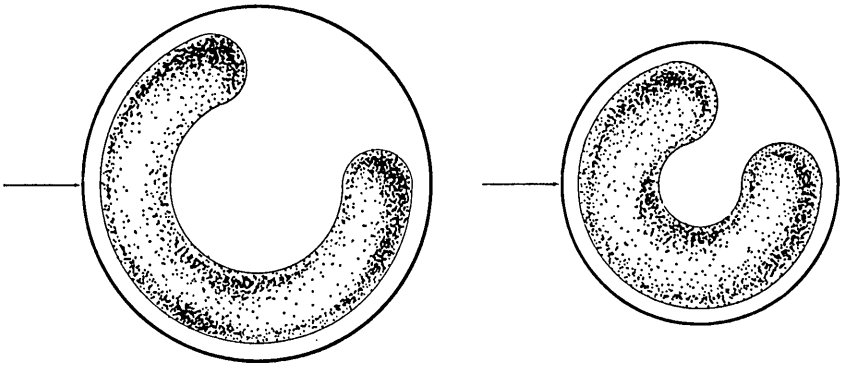
The other possibility is that the erythrocyte membrane itself may have altered viscous properties in diabetes. The elastic properties of the erythrocyte membrane are produced by the spectrin-actin complex, a protein network located at the inner surface of the plasma membrane.[13] This network is responsible for restoring the discoid shape of red cells,[14] and viscous resistance to deformation is produced by its interaction with other membrane proteins and lipids. Erythrocyte membranes isolated from diabetics with recent episodes of poor control are stained by hemoglobin, suggesting that changes in this area may occur in diabetes,[15] but our findings indicate that abnormal red cell deformability is not limited to diabetics with recent poor control. An abnormality of erythrocyte membrane lipids could also be produced by the metabolic alterations in diabetes. Platelet phospholipids have already been found to be abnormal in juvenile diabetes.[16]

Increased viscosity of diabetic erythrocytes would have little effect in large vessels, where the deformability of the individual red cells is not a major factor in flow. But as the red cell enters smaller and smaller vessels in the microcirculation, its ability to deform becomes important. In vessels of 8 μ or less, the red cells must change in shape. The increased viscosity reduces the rate of cell deformation, unless the force on each red cell is raised by an increased pressure gradient. The diabetic circulation would have such a requirement only during maximum flow.

Impairment of maximal blood flow in exercising muscle would be expected, and such an impairment in the maximum exercise ability of diabetics has been reported.[17,18] Diabetics also have increased blood pressure with maximal exercise compared with non-

FIGURE 3

Shown are two diagrams of the cross-sectional configuration of red cells in a micropipet. Each red cell is shown in the configuration described by Rand.[9] (The change in diameter of the tube at each end of the migration path has been markedly exaggerated.) The plasma layer between the red cell membrane and the wall is shown. During each cycle of movement in the pipet, the red cell expands and contracts while maintaining the same general shape.



diabetics, possibly an adjustment to the red cell problem and the alterations produced by diabetic microangiopathy.

As in movement toward the micropipet tip, the diabetic erythrocyte, entering into a vessel area into which it is compressed, would exert more pressure on areas of the vessel wall because of its increased resistance to shape change (figure 4). This may stimulate basement membrane thickening if, as suggested by Williamson et al. in studies of the giraffe,[19] increased pressure causes basement membranes to thicken.

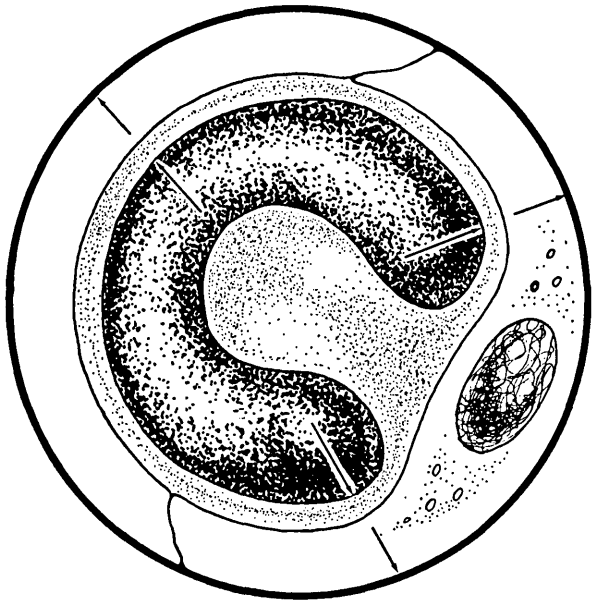


FIG. 4. Shown is a deformed red cell in a capillary. Pressure produced by deformation of the red cell would be exerted across the fluid layer and endothelial cell to the capillary basement membrane at and between the points shown by the arrows wherever the curved membrane is close to the wall. Because erythrocyte viscous resistance is increased in diabetes, the pressure exerted would be increased mainly where the vessel narrows or curves. Since different erythrocytes may pass in different planes, any increase in pressure could be distributed over the entire circumference or any part, depending on local anatomic features.

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