# Critical Cell Volume and Shape of Bovine Erythrocytes

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Abstract. The relationship between erythrocyte shape and the critical cell volume was investigated Agents able to increase the critical cell volume induced three main stable shapes of erythrocytes discocytic, stomatocytic, and echinocytic. The absence of correlation between shape and critical cell volume under isoosmotic conditions suggests that relative differences between the surface areas of the inner and the outer leaflet of the cell membrane do not influence the critical volume of a cell

Key words: Bovine erythrocytes — Critical cell volume — Cell shape

## Introduction

Under physiological conditions the red cells of most mammals have a steady biconcave shape Many agents, such as local anesthetics and tranquilizers, produce other stable erythrocyte shapes (Sheetz and Singer 1974, 1976) The chemically induced shape transformation were interpreted by the bilayer couple hypothesis (Sheetz and Singer 1974, 1976, Svetina and Zekš 1983), and changes in shape were explained by an increased surface area of the outer or the inner leaflet of the membrane bilayer, caused by a selective intercalation of amphipatic molecules The combined concept of membrane spontaneous curvature and the bilayer couple hypothesis, proposed by Svetina and Zekš (1983) lead to the conclusion that the critical cell volume of erythrocytes depends on the relative difference between the areas of the outer and the inner bilayer leaflet In consequence, the erythrocyte shape would be expected to correlate with alterations of the critical cell volume (Seeman et al 1969b, 1969c) To test the hypothesis of Svetina and Zekš, the correlation between erythrocyte shape and the critical volume was studied using four different approaches 1 By changing the glycolytic activity of erythrocytes

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by phosphate (Rose et al. 1964) and arsenate ions; 2. By heating erythrocyte suspensions to 49°C (Anderson and Marchesi 1985); 3. By the influence of vanadate ions on the erythrocyte shape (Ferrel and Huestis 1984; Backman 1986); 4. By the influence of DIDS (4,4'-diisothiocyanostilbene-2, 2'-disulphonic acid) on the interaction of spectrin with the lipid bilayer (Hsu and Morrison 1983).

## **Materials and Methods**

### Erythrocytes

Fresh, heparinized bovine blood was centrifuged at 2 700 x g for 10 min at 4 °C, plasma and buffy coat were removed, and the red cells were washed three times with an isotonic (310 mOsmol/l) solution A containing: 138 mmol/l NaCl; 5 mmol/l KCl; 1.5 mmol/l MgCl<sub>2</sub>; 10 mmol/l Tris-HCl; pH 7.4. The chemicals were of analytical grade.

### Cell treatment

a) Heating: Glass tubes containing the red blood cells suspensions (25% hematocrit) were heated in a water bath at  $49.0\pm0.1$  °C for 10 min (Rakow and Hochmuth 1975). The samples reached the temperature within 2 min. After the incubation they were cooled down to room temperature by swirling the tubes in tap water. b) Activation of glycolysis: The cell suspension (5% hematocrit) was incubated at 37 °C for 2 h in solution B containing: 109 mmol/l NaCl; 5 mmol/l KCl; 1.5 mmol/l MgCl<sub>2</sub>; 20 mmol/l Na<sub>2</sub>HPO<sub>4</sub>/NaH<sub>2</sub>PO<sub>4</sub>; 10 mmol/l glucose; pH 7.4. c) Metabolic starvation: The erythrocytes suspension (5% hematocrit) was incubated at 37 °C for 4 h in solution C containing: 114 mmol/l NaCl; 5 mmol/l KCl; 1.5 mmol/l MgCl<sub>2</sub>; 20 mmol/l Na<sub>2</sub>HPO<sub>4</sub>/NaH<sub>2</sub>PO<sub>4</sub>; pH 7.4, or in solution D which contained: 125 mmol/l NaCl; 10 mmol/l Na<sub>2</sub>HAsO<sub>4</sub>; 10 mmol/l Tris; pH 7.4 adjusted with HCl. d) Vanadate treatment: The erythrocytes were incubated at 37 °C for 2 h in solution E which contained: 133 mmol/l NaCl; 5 mmol/l KCl; 1.5 mmol/l MgCl<sub>2</sub>; 1 mmol/l Na<sub>3</sub>VO<sub>4</sub>; 10 mmol/l Tris-HCl; 10 mmol/l glucose; pH 7.4; hematocrit 5%. Orthovanadate was from Sigma. e) DIDS treatment: The suspension of red blood cells (25% hematocrit) was incubated at 37  $^{\circ}$ C for 1 h in solution F containing: 143 mmol/l NaCl; 5 mmol/l KCl; 1.5 mmol/l MgCl<sub>2</sub>; 20 µmol/l DIDS; 5 mmol/l HEPES; pH 7.4. All samples were washed twice after incubation with ten volumes of solution A.

### Critical cell volume.

The critical cell volume was calculated from van't Hoff's law modified for erythrocytes (Dick 1959), according to the formula:  $V_c = c/\pi_n + b$ , where c is the sum of products of osmotic coefficients and the numbers of moles of internal solutes,  $\pi_n$  is the cell osmotic fragility, b is the volume of osmotically non-active part of the erythrocyte. The osmotically non-active part of the erythrocyte volume and the amount of the intracellular solutes were obtained by a mathematically modified microhematocrit method, described in detail in previous papers (Mosior and Gomułkiewicz 1985; Mosior et al. 1988). The measure of osmotic fragility was the mean osmolarity of solutions in which hemolysis occurred (Mosior and Gomułkiewicz 1985). The hemolysis was carried out in hypotonic NaCl or 75% NaCl + 25% KCl solutions buffered with 5 mmol/l phosphate (Na<sub>2</sub>HPO<sub>4</sub>/NaH<sub>2</sub>PO<sub>4</sub>), pH 7.4. The relative isoosmotic volume of the erythrocytes was determined from the absorbances of hemoglobin released from control and modified erythrocytes in suspensions with identical hematocrits (Mosior and Gomułkiewicz 1985). The volume of the solution

trapped between packed cells was estimated by the hemoglobin method (Mosior et al. 1988).

#### Red cell morphology.

The red cells were suspended in plasma to 1% hematocrit and then examined under a light microscope. Some cell samples were fixed in 1% glutaraldehyde, post-fixed in OsO<sub>4</sub> and dehydrated in ascending ethanol series. After drying and coating they were photographed under a scanning electron microscope.

	Isoosmotic volume	Amount of intracellular solutes	Osmotically non-active volume	Osmotic fragility	Critical cell volume
				π <sub>h</sub>	
	%	%	%	%	%
B C D E F	$101.6 \pm 0.7 \\98.7 \pm 1.0 \\97.7 \pm 1.0 \\98.9 \pm 0.9 \\101.1 \pm 0.7 \\100.0 \pm 0.5 \\100.0 $	$103.4 \pm 3.0 \\99.8 \pm 3.8 \\90.1 \pm 1.8 \\93.4 \pm 2.2 \\103.7 \pm 2.9 \\101.4 \pm 0.9 \\101.4 $	$99.1 \pm 3.5 97.3 \pm 4.6 107.0 \pm 2.4 108.5 \pm 3.0 97.3 \pm 5.5 2020 \pm 4.0 2010 \pm 5.5 2020 \pm 4.0 2010 \pm 5.5 2020 \pm 5.5 \\2020 \pm 5.5 \\20$	$99.2 \pm 0.6 95.5 \pm 2.2 94.6 \pm 2.1 98.6 \pm 0.4 98.5 \pm 1.1 100 + 100 + 100 100 + $	$102.4 \pm 1.1 102.6 \pm 1.5 99.1 \pm 0.8 98.8 \pm 0.7 102.5 \pm 0.9 97.5 \pm 1.2 99.1 \pm 0.9 97.5 \pm 0.9 \\97.5 \pm 0.9 \\9$

Table 1. Effect of cell modifications on the osmotic properties of bovine red blood cells

The figures shown are means (n = 8) and confidence limits for the level of confidence 1 - P = 0.95. All values are related to the control values (solution A) which are (mean  $\pm$  SD): osmotically non-active volume  $b = 44, 5 \pm 2.5\%V_i$ ; critical cell volume  $V_c = 144.1 \pm 5.5\%V_i$ ; osmotic fragility  $\pi_h = 55.8 \pm 2.8\%$ , where 100% corresponds to the osmolarity of 310 mOsmol/l;  $V_i$  is the isoosmotic volume of the cell. Since osmolarity and cell volume values are in relative units, the amount of the intracellular solutes per unit isoosmotic volume c = (1 - b)/100 (for the control cells only). The cell treatments and the modification agents were as follows: B, incubation at 49 °C for 10 min; C, incubation at 37 °C for 2 h in solution containing 20 mmol/l phosphate and 10 mmol/l glucose; E, incubation at 37 °C for 4 h in solution containing 10 mmol/l arsenate; F, incubation at 37 °C for 2 h in solution containing 1 mmol/l orthovanadate and 10 mmol/l glucose; G, incubation for 1 h in solution containing 20  $\mu$ mol/l DIDS.

## Results

The values of the calculated critical cell volume (CCV), and of all parameters used for the calculations, are shown in Table 1. relative to the corresponding parameters for control cells. The values of the critical and the osmotically non-active volumes were multiplied by the relative isoosmotic volume of the modified cells to allow a direct comparison of the data

The incubation with phosphate ions and glucose, or with orthovanadate ions, and a 10 min incubation at 49 °C, caused a 2-3% increase of the critical cell volume of the erythrocytes DIDS treatment of the cells decreased CCV by a similar percentage Incubation with phosphate ions decreased the CCV by about 1% Since CCV was computed from the osmotic fragility, a possible effect of the hemolysis-protective potassium efflux on that parameter (Jay and Rowlands 1975, Seeman et al 1969a) was also checked For that purpose, the cells were hemolysed in two series of hypotonic solutions one with only Na<sup>+</sup> ions, and the second one containing also 25% K<sup>+</sup> ions (in the intracellular solution of bovine erythrocytes, potassium ions make up no more than 25% of all cations (Bartosz et al 1981)) The hemolysis curves for control and all modified cells were identical in both solutions Since the shifts of the hemolysis curves, due to cell modifications, were similar, that for vanadate treated cells is shown as an example only (Fig 1) The volumes



Figure 1. The effect of vanadate on the hemolysis of erythrocytes in NaCl and NaCl+KCl solutions

The hemolysis curves, identical for control samples (squares), were shifted upon vanadate treatment of the erythrocytes towards smaller fragilities (circles) The shift was similar in both NaCl (filled symbols) and in NaCl+KCl solutions The error bars do not exceed the size of the symbols

of the trapped extracellular solution did not change statistically significantly in all samples after treatments, being within 22 - 28% of the isoosmotic cell volume. The erythrocyte shapes as seen under a microscope are shown in Fig 2 Incubation at 49 °C caused a transformation to a specific, conical stomatocytic shape (Fig 2b)



Figure 2. The effect of cell treatments on erythrocyte shape.

All samples were washed twice with solution containing 138 mmol/l NaCl; 5 mmol/l KCl; 1.5 mmol/l MgCl<sub>2</sub>; and 10 mmol/l Tris-HCl; pH 7.4, fixed with 0.5% glutaraldehyde, which itself did not alter the erythrocyte shape, and observed under an electron scanning microscope. A, control cells; B, cells incubated for 10 min at 49 °C; C, cells incubated for 2 h at 37 °C in solution containing 1 mmol/l orthovanadate and 10 mmol/l glucose; D, cells incubated for l h at 37 °C in solution containing 20  $\mu$ mol/l DIDS.

The cells incubated in the presence of phosphate ions, with or without glucose, retained the biconcave shape, with a slight crenation in the glucose-free medium. Arsenate caused a more pronounced crenation. The vanadate treatment led to the formation of strongly crenated echinocytes (Fig. 2c). DIDS treated cells formed ball-like echinocytes with narrow protrusions (Fig. 2d).

# Discussion

# Critical cell volume

CCV of erythrocytes was computed from van't Hoff's law, modified for erythrocytes (Dick 1959, Mosior and Gomułkiewicz 1985), the applicability of which was proved up to the spherical cell shape (Richieri and Mel 1985) The absence of hemolysis-protective potasium efflux, as well as independent measurements of osmotic fragility and of the amounts of internal solutes, allow to conclude that the observed changes in CCV were due to alterations of the membrane extensibility The observed changes in CCV (1 - 3%), are small in comparison with the CCV value itself, but they are large when compared with the elastic increase of the cell volume, which is equal to 4 - 9% of the spherical volume of erythrocytes (Evans et al 1976, Massaldi et al 1983) The influence of changes in osmotic fragility, due to alterations of the composition of the internal solution, on CCV, was tested by measuring both the isoosmotic and the osmotically non-active volumes, as well as the amount of the internal solutes Thus, alterations of membrane extensibility most probably are involved in the observed changes in CCV

## The erythrocyte shape

Erythrocytes with an enlarged critical cell volume exist in all three basic stable shapes of the cell under isoosmotic conditions The increase in CCV, and the transformation to conical shape after incubation at 49°C, were probably caused by spectrin denaturation, taking place at this temperature (Brandts et al 1977) Such a treatment of cells significantly changes the elastic shear modulus and the microviscosity of the cell membrane (Evans et al 1976, Kucera et al 1986), how ever without its fragmentation (Rakow and Hochmuth 1975) The crenation of the erythrocyte induced by vanadate was probably associated with the phosphorylation of phosphatidylinositol 4,5-biphosphate and phosphatidic acid (Ferrel and Huestis 1984, Kucera et al 1986) Despite a reduction of the inner surface area of the bi layer, caused by degradation of phosphatidylinositol, the CCV of the erythrocytes increased (Mosior et al 1992) This effect may be explained by the dependence of the lipid bilayer – membrane skeleton interaction on the phosphorylation state of phosphatidylinositol Degradation of phosphatidylinositol lipids yields the dissociation of the glycophorin – band 4 1 complex (Anderson and Marchesi 1985), which my result in a larger membrane extensibility (Mosior et al 1988, 1990) The in cubation of erythrocytes with phosphate ions and glucose caused CCV to increase without any visible alterations of the normal biconcave shape of the cells The metabolic starvation, or arsenate treatment caused a slight crenation of erythro cytes DIDS induced a transformation to a crenated sphere, decreasing the CCV contrary to vanadate, which also caused a crenation of the cells DIDS molecules bind to band 3 protein of the erythrocyte membrane (Cabantchik and Rothstein

1972) and, unlike the anions used in this study, interact directly with the lipid protein matrix of the cell However, DIDS does not significantly increase the area of the membrane surface, while decreasing the CCV (Mosior et al 1992)

The results discussed above lead to the conclusion that in erythrocytes shape alterations do not correlate with the critical cell volume, in contrast to simple expectations drawn from the hypothesis proposed by Svetina and Zekš (1983) The elastic bending energy of erythrocyte membrane is several orders of magnitude lower than the energy of elastic compressibility (Evans and Skalak 1980) Therefore, the stretched erythrocyte membrane is always ruptured after the cell has reached a spherical shape Evans et al (1976) observed that small vesicles attached to the membrane surface recombined with the cell membrane by incremental jumps when the membrane was being stretched The change of the relative difference of the surface area between the inner and the outer leaflet of the erythrocyte membrane bilayer, which induces the shape transformations of the cells, does not alter the cells critical volume

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