

BASIC RESEARCH

Shape and Volume Restoring Phenomena in Human Erythrocyte Suspension under Low Ion Strength Conditions

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Abstract

In this study, the earlier used method to measure the dynamics of shape changes in red blood cells (RBCs), based on an analysis of light fluctuations in the suspension, was modified to allow for the simultaneous recording of cell volume changes after appropriate recalculation of the raw absorbance and shape index data. With this improved methodology, we investigated the morphological and volume responses triggered by cell environment changes. In a low ionic strength medium (LIS), the characteristic triphasic shape changes (morphological response, MR) were accompanied with a gradual shrinking of the cells without any re-swelling phase. The addition of hyperosmotic NaCl during the terminal MR phase restored the discoid RBC shape inducing cell swelling resembling regulatory volume increase. The cell volume was greater than that before salt addition; however, it was lower than the initial isotonic cell volume. This re-swelling phase was inhibited by the external DIDS, acetazolamide and bicarbonate, and was slightly dependent upon pH ranging from 5 to 7.2. The analysis shows that chloride-induced re-swelling cannot be directly explained by the reversion of OH⁻ or HCO₃⁻ gradients which drive the Cl⁻ inside the cells against the concentration gradient, and indicates the significant role of the external bicarbonate ions in shape and volume responses in LIS.

Key words: red blood cells; morphological response; low ionic strength; DIDS; aluminum.

Abbreviations

HBS, HEPES buffered saline; **PS**, physiological saline; **RBC**, red blood cell; **hRBC**, bicarbonate-enriched red blood cell; **MR**, morphological response; **SI**, shape index; **SSS**, standard sucrose solution; **LIS**, low ionic strength; **NSVDC**, non-selective voltage dependent channel; **OUW**, osmotically unresponsive water; **AcZA**, acetazolamide; **BzA**, benzalkonium chloride; **RVD**, regulatory volume decrease; **RVI**, regulatory volume increase; **CAII**, carboanhydrase II; **AE1**, anion exchanger 1 (band 3); **VRS**, volume responsive system; **SRS**, shape responsive system.

Introduction

The atypical properties of the red blood cells in low ionic strength solutions (LIS), where NaCl is substituted isosmotically by sucrose, are still not well understood. LIS and nonelectrolytic solutions, particularly sucrose, are the media in which the red cells change their ionic and osmotic states. These solutions have been used to study the anion and cation fluxes [1-6] and the

morphological changes [7] of the RBCs [8]. However, the nature of the molecular mechanisms leading to the activation of some normally dormant transport pathways [1-7, 9-13] and the basis of the characteristic triphasic shape changes in a sucrose solution [8,14] are still obscure. In the earlier studies we showed that the terminal stomatocytic RBC shape in a sucrose solution can be reversed to a discoid shape by the extracellular Cl⁻ concentration increase accompanied by apparent cell swelling [8].

The aim of the present study was to investigate, in greater detail, the relationship between the RBC shape and volume regulatory mechanisms in LIS, including the role of protons, chloride and bicarbonate. We conclude that the RBC volume responses in LIS cannot be satisfactorily explained using the existing models.

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Materials and Methods

N-(2-Hydroxyethyl)piperazine-N'-2-ethanesulfonic acid (HEPES) and 4,4'-diisothiocyanato-2,2'-stilbene-disulphonic acid (DIDS) was purchased from Serva, and sucrose from Merck. All other reagents and AlCl_3 were obtained from major suppliers and were of the highest purity available. The stock solution of benzalkonium chloride (BzA; pharmaceutical mixture, consisting of a mixture of alkylbenzyltrimethylammonium chlorides of various even-numbered alkyl chain lengths, Fluka, Germany) was prepared with concentration of 0.4 mg/ml.

Preparation of Erythrocytes

Human blood of informed volunteers was collected in vacuum vacutainer tubes with EDTA as anticoagulants on the day of the experiment. After free blood sedimentation, 0.225 ml of the red blood cell pellet was transferred into and washed once with 5 ml of physiological saline (PS) (150 mM NaCl, 3,000 g, 2 min) and then once again with 5 ml of HEPES buffered saline HBS (150 mM NaCl, 5 mM HEPES, pH 7.4, 3000 g, 1 min). Excess supernatant after the centrifugation was removed to leave ~1.5 ml final volume of red blood cell stock suspension with hematocrit about 17% which, if not indicated otherwise, was allowed to stay for 1-2 hours at room temperature before measurements to avoid initial drift in cell properties [14]. To obtain a bicarbonate enriched RBCs (bRBCs) the pellet of cells in HBS was divided into equal portions and concentrated bicarbonate solution (0.1 M) was added to one portion to get 10 mM final concentration. The same amount of HBS was added to other control portion of RBCs. To treat RBCs by DIDS, standard stock suspension was incubated at room temperature for 30 min in the presence of 20 μM DIDS and used as is.

Measurement of dynamics of RBC shape and volume changes

Morphological response of the cells suspended in a standard sucrose solution (0.3 M, pH 5.8-6.2, without buffer, SSS) was monitored using homemade SA-01 shape-meter/aggregometer which allows, besides measuring optical density or light transmission, assessment of light fluctuations carrying information on cell shape [15]. The shape index (SI) was calculated following the previously described protocol [8,15] from the equation: $SI = k \cdot D$, where k is a constant depending on amplification factor and meter calibration and D is a mean value of light amplitude fluctuations, averaged in 1-s time intervals. The calibration factor k allows formation of SI scale reflecting the erythrocyte discoidal-spherical shape factor (1.00 for discs and 0.06 for spheres). Erythrocytes (6-8 μl of stock suspension) were placed into a cylindrical (diameter 10 mm) glass cell containing 2 ml of HBS so that the initial optical density was 0.30 ± 0.01 , which corresponds to cell concentration of $6 \cdot 10^6/\text{ml}$. The cell suspension was stirred on a magnetic stirrer at 600 rpm. Morphological changes were monitored using an equally dense cell suspension in SSS. In other experiments additional chemicals (0.5-100 μl of stock solutions with the same pHs as SSS) were added directly to the cells up to the desired final concentration. Data presented in the figures are typical from 3 to 5 independent experiments carried out with blood from different donors. All experiments were performed at room temperature (20-24°C).

Volume changes were recalculated from raw absorbance and SI data using formula A7 as described in the Appendix.

Results

Dynamics of volume and shape changes during morphological response (MR) and effects of anions and DIDS

As shown elsewhere [14], variability in the absorbance time course and SI changes of red blood cells in standard sucrose solution (SSS) are noted (all abbreviations used in this paper are given in the abbreviation list). Despite the absolute absorbance values and SI variability, the recalculated volume changes in most cases demonstrate rather conservative, and often, equal behavior. As demonstrated in Figs. 1 and 2, shrinkage usually produces a smooth, close to a single exponential, time course with maximal volume changes occurring within the first 60 s of process where the volume reaches approximately 88% of the initial cell volume. After 5 min this value drops to $84 \pm 2\%$. At the peak of MR (approximately 20 s), where the cells restore their discoid shape, they have a reduced volume of 94%.

Fig. 1

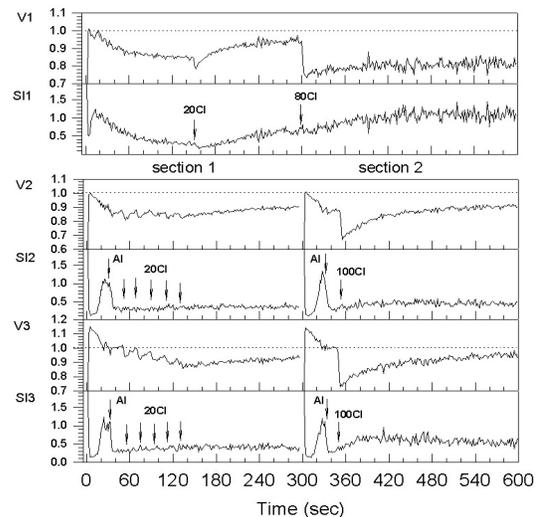


Figure 1.

Dependence of the calculated relative cell volume (V) and the corresponding values of the shape index (SI) on time during the morphological response (MR) in sucrose solutions. The pair ($V1$, $SI1$) shows MR in the standard sucrose solution (SSS) and the effect of chloride added at some points in time on the subsequent shape and volume dynamics. The pair ($V2$, $SI2$) shows an effect of chloride and aluminum (final concentration 20 μM , label Al) in SSS and the pair ($V3$, $SI3$) in hypotonic sucrose (0.23 M), respectively. The arrows show a moment of reagent addition. Labels 20Cl, 80Cl and 100Cl show the addition of 20, 80 and 100 μl of NaCl (2 M) in 2 ml of cell suspension, respectively. In $V2$ and $V3$ the solutions additionally contained 0.4 $\mu\text{g}/\text{ml}$ BzA. Here and in other figures the whole experimental scan lasted 600 s can be divided on sections corresponding to separate experiments with new portion of cells. For example, section 1 and section 2 show individual tracks each lasted 300 s obtained immediately after placing the cells into the medium. Therefore, the end of the experiment in the section 1 (300 s) corresponds to the beginning of the next experiment in the section 2, i.e. time zero. The same is true if the number of sections is more than two.

The value concurs with the earlier estimate that the cells in state S_2 have a slightly reduced volume [8]. The data demonstrate that the improved technology, that converts the raw absorbance

and SI data into volume changes, produces results showing a rather high quantitative sensitivity (~2%) in assessing the relative volume changes during MR.

Fig. 1 shows that addition of NaCl at terminal phase of MR produces pronounced shape and volume restoration of shrunken and stomatocytic cells. In the presence of BzA and Al^{3+} , that inhibited shape changes, an addition of concentrated NaCl produced a small hypertonic shrinkage followed by a volume recovery.

The dynamics of these processes was similar in isosmotic as well as in hyposmotic sucrose solutions (Fig. 1, V2 and V3). In both cases the cells reached the corresponding final volume which did not depend on the way NaCl was added (a small bolus or all-in-one, Fig. 1, V2, V3, sections 1 and 2). Interestingly, despite the increase in osmolarity produced by the addition of salt, the final cell volume after NaCl addition was greater than before the addition. Thus, the cells not only restore an initial

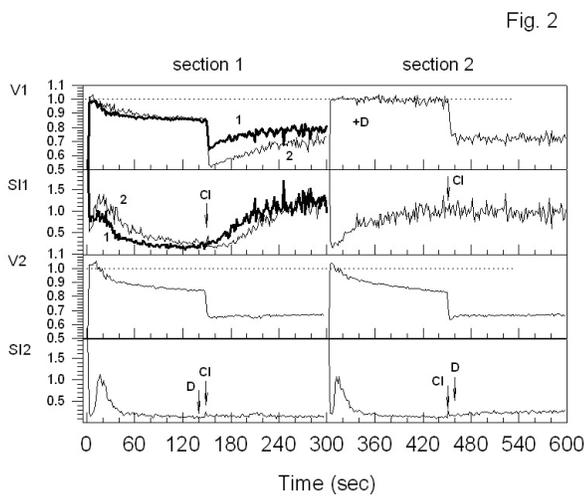


Figure 2.

Dependence of calculated relative cell volume and SI on time during MR in SSS and the effect of DIDS on chloride-induced shape and volume changes. The arrows with the Cl label show the time of the addition of NaCl at the final concentration of 49 mM, except for curve 2, which corresponds to NaCl concentration of 95 mM. The arrows with label D show a moment of addition of DIDS at final concentration 2 μ M. Labels with (+) indicate that the reagents were added to SSS prior the cells.

sucrose-induced shrinkage, but in addition overcome a shrinkage induced by rather strong hypertonicity. The re-swelling process takes place at any NaCl concentration exceeding 10 mM and can be inhibited by DIDS as it is shown in Fig. 2.

At concentrations lower than 10 mM (not shown in the Fig.), chloride is also able to inhibit the sucrose-induced shrinkage and cause some restoration of volume and shape. However, lowering the chloride concentration decreased the degree of restoration (not shown), demonstrating that the restoration process is concentration dependent. When DIDS was included in the sucrose solution (Fig. 2, V1, section 2), it completely inhibited the sucrose-induced shrinkage but had no effect on hypertonic NaCl-induced shrinkage. This is normal because DIDS does not inhibit the water channels. However, it must be noted, that the final cell volume is lower when DIDS is added before (or after) NaCl in phase 3 of the standard MR compared with MR in the

presence of DIDS (phase 1) (Fig. 2, V1, section 2; V2, sections 1 and 2). The data demonstrate that volume changes in the hypertonic mixed NaCl/sucrose solution are reversible. The same is observed in an isosmolar solution containing 50 mM NaCl. The cells do not substantially change their volume when placed directly in the solution (Fig.3, V1, section 2) and they recover the original isotonic volume after the addition of NaCl at the same medium osmolarity (Fig.3, V1, section 1).

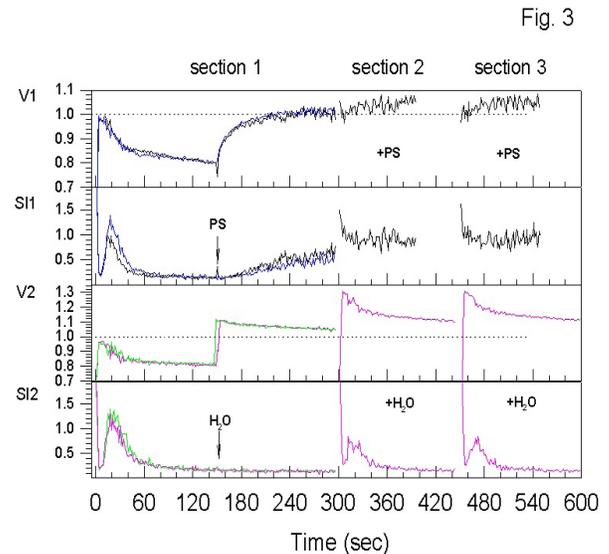


Figure 3.

Dependence of the calculated relative cell volume and SI on time during MR in SSS and the effect of the addition of 1 ml PS (V1) or distilled water (V2) to 2 ml of cell suspension. Arrows show moment of addition of PS or H_2O . Labels with (+) indicate that test solutions were added to SSS prior the cells. Volume data were corrected for dilution factor. Two repeated performances of the experiments with (+) are separated in distinct sections 2 and 3.

When NaCl was substituted with distilled water to produce hypotonicity, the cell predictably swelled rapidly that followed by a less visible shrinkage (Fig.3, V2, section 1).

Under hyposmotic conditions the cells increased their volume and demonstrated the same time-course of volume changes as normal (Fig. 3, V2, section 2). The results listed in Figs. 1, 2 and 3 suggest that the characteristic $t_{1/2}$ shrinkage and re-swelling time shows similar values for all the cases. It assumes that the basic mechanisms regulating volume changes under the conditions where the extracellular chloride content varies are similar in nature. The mechanism probably includes a redistribution of anions and protons between the cells and the medium. However, two lines of evidence prompt the exclusion of the possibility that Cl^- -induced re-swelling reflects the cell swelling induced by the low pH of the medium due to Cl^-/OH^- exchange in a nonelectrolytic solution.

i) The present experiments were performed with low hematocrit red blood cell suspension (~0.06%) where a drop in external pH during cells exposure in LIS was about 0.2 unit (measured with pH electrode). This relatively small change in pH could hardly induce a dramatic volume compensation followed by 95 mM NaCl addition, as shown in Fig. 2.

ii) When the external pH was changed by addition of NaOH or HCl to check the role of pH in the cell-volume recovery, the results shown in Fig. 4 were obtained. The control cells demonstrated the classical swelling in acid and shrinkage in base

Fig. 4

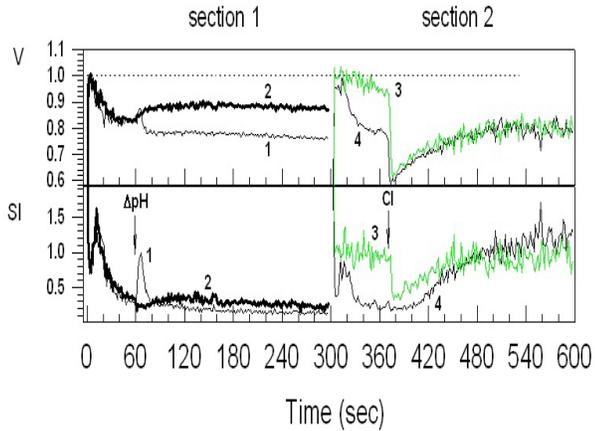


Figure 4. Effects of chloride and pH on the calculated relative cell volume and SI changes during MR in SSS. Tracks 1 and 4 correspond to basic pH and tracks 2 and 3 to acid pH, respectively. The arrows show moment of reagent addition. Labels Cl show addition of 100 μ l of NaCl (2 M) in 2 ml of cell suspension. Label Δ pH show moment of addition of NaOH or HCl in order to increase or decrease medium pH in 1-1.2 unit. Section 2 corresponds to the case where the same pH changes of SSS were performed before addition the cells.

solutions (Fig. 4, section 1). However, any variation in external pH (increase or decrease) did not change the re-swelling response of the cells after chloride application (Fig. 4, section 2) but strongly influenced the dynamics of MR visualized in SI tracks.

Base pH increased, whereas acid pH reduced, the rate and the degree of sucrose-induced volume changes confirming the important role the hydroxyl gradient plays in the stimulation of Cl^-/OH^- or $\text{Cl}^-/\text{HCO}_3^-$ exchange during the initial shrinkage. Quantitatively, the same results were obtained when the pH of the sucrose solution was changed 10 s after or before the addition of NaCl (data not shown). The data clearly illustrate the presence of a weak pH-dependent volume recovery process that causes the re-swelling of osmotically shrunken cells.

Effect of bicarbonate and acetazolamide on morphological response

The data shown in Fig. 5 illustrate that the bicarbonate added after the re-swelling phase causes additional monophasic cell shrinkage. Biphasic shrinkage, consisting of fast and slow components, was observed when the bicarbonate was added before NaCl (Fig. 5, V1, section 2). Only the slow component was fully inhibited by the carbonhydrase inhibitor acetazolamide in both normal and bicarbonate-enriched bRBC (Fig. 5, V1, V2, sections 2).

Acetazolamide inhibited shape restoration and Cl^- -induced re-swelling in both cell types (Fig. 5, V1, V2 sections 1, curves 2). However, its presence did not affect the final volume of the cells after the subsequent addition of the bicarbonate. By contrast, the

final volume of cells was significantly larger in the presence of the acetazolamide compared with the control if the bicarbonate was added before the NaCl (Fig. 5, V1, V2 sections 2, curves 4). Besides, the acetazolamide significantly accelerated the morphological changes during MR and eliminated any subsequent NaCl-induced (or bicarbonate) shape changes which are clearly visible in the control samples. In the presence of the extracellular bicarbonate, the cells rapidly reduced their volume down to 0.8 of the original volume. Neither DIDS nor acetazolamide was able to inhibit this fast shrinkage (Fig. 5, V3). Interestingly, by contrast to pure SSS, where the DIDS fully inhibited re-swelling (Fig. 2, V1, section 2) in the SSS-containing bicarbonate and DIDS (or acetazolamide), the addition of NaCl continues to induce small re-swelling (Fig. 5, V3). Thus, it appears that the inhibition of re-swelling in the presence of the bicarbonate is not a true inhibition of swelling. Rather, it represents a side effect of the opposite of bicarbonate-induced shrinkage, as the addition of bicarbonate at the end of the re-swelling phase induced an abrupt sphering and more prolonged cell shrinkage. The data shown in Fig. 5 demonstrate that the cells may have different steady-state volumes and shapes in media containing the same final composition. Thus, these data strongly confirm that the amplitude of volume changes and the final cell volume at a given osmolarity is not a constant, and depends upon the composition of the medium and the prehistory of the cells (e.g., whether the cells were swollen or shrunken before the osmotic challenge or if DIDS or acetazolamide were present).

Fig. 5

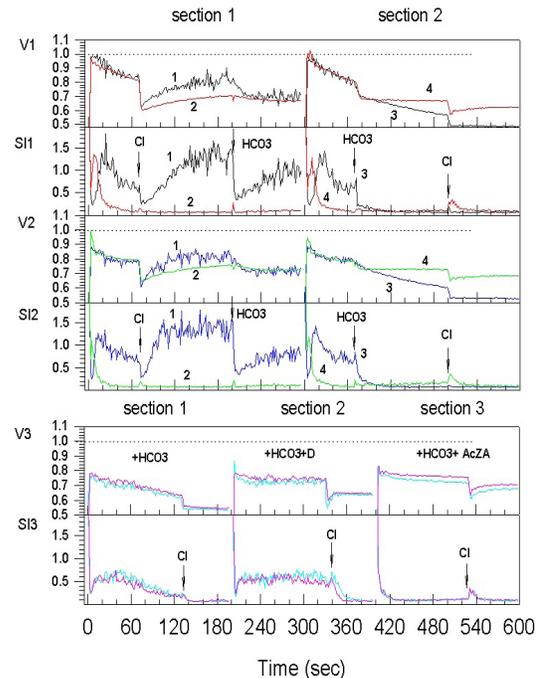


Figure 5. Effect of chloride and bicarbonate on the calculated relative cell volume and SI changes during MR in SSS. The arrows show a moment of reagent addition. Labels Cl show addition of 100 μ l of NaCl (2 M), label HCO_3^- – 10 μ l of NaHCO_3 (0.1 M, final concentration 0.5 mM) in 2 ml of cell suspension. Labels with (+) indicate that the reagents were added to SSS prior the cells. Final concentrations of DIDS and AcZA were 2 and 180 μ M, respectively. Data shown in V1 and V2 correspond to normal cells and bRBC, respectively in which tracks 1 and 3 were obtained in pure SSS, whereas tracks 2 and 4 were obtained from SSS containing AcZA. In V3 the tracks are, in fact, identical and one track corresponds to the normal cells and other one to bRBC.

Discussion

The data presented indicate that human red blood cells undergo pronounced re-swelling resembling classical RVI in hyperosmotic medium (Figs. 1 and 2) and they slightly reduce the volume in hyposmotic ones (Fig. 3, V2). However, the responses are not, in fact, RVI and RVD because they too take place in an isosmotic medium (Fig. 3). The mechanism of these responses is, therefore, suggested to be different from that of RVD and RVI.

In RBC research, it is generally accepted that under LIS conditions, many cell properties dramatically alter, especially those related to ionic and nonelectrolytic transport [1-6,9-13,16]. They also change shape and demonstrate a morphological response [8,14]. It was suggested earlier, that when the Cl^- concentration in the medium is reduced, the depolarized cells will initially lose the intracellular Cl^- -mediated by the Cl^-/OH^- exchange with concomitant obligate water efflux resulting in shrinkage [13]. Secondary, the KCl efflux will stimulate further shrinking. Our kinetic data relating to the earlier phase of the process during which K^+ release is negligible [5,9] demonstrate a major volume reduction within the first 30-40 s to approximately 0.88 of the initial isotonic volume followed subsequently by a slower shrinkage. This is in a close quantitative agreement with the data reported in [16]. To avoid fast shrinkage, Kumerow et al. [13] used hypotonic sucrose solution containing 10 mM Tris in which the immediate alkalization of the cytosol was noted. Both fast initial shrinkage and alkalization were attributed to the AE1-mediated Cl^-/OH^- exchange. In another work by Bisognano et al. [3], the response of the cells incubated in physiological saline on hypertonic sucrose was biphasic, resulting in fast hypertonic shrinkage and gradual further shrinkage. The latter was accompanied with proton efflux indicating the HCl cotransport or Cl^-/OH^- exchange. The data concur with the viewpoint that an initial fast shrinkage in the sucrose medium is mediated by the Cl^-/OH^- exchange, whereas a subsequent slow shrinkage is already due to the K^+ efflux. Our data extend these observations to the RBC volume dynamics. Monophasic shrinkage alone was found when the K^+ efflux was stimulated by CCCP and valinomycin [17], whereas biphasic ones occurred in the presence of bicarbonate (Fig. 5, V1, section 2 and V3, section 1). The data also confirm the earlier observations that the Cl^-/OH^- exchange-mediated shrinkage and cell alkalization [13] are inhibited by DIDS, attributing the effect to AE1. Considering the molecular basis of the volume-restoring phenomenon, it is unclear which component is loaded during the Cl^- -induced cell re-swelling in the sucrose solution. Sucrose could not be the culprit as it is well known that the sucrose is impermeant for red cell membranes [18]. The Na^+ could not be held responsible either, because identical results were obtained if KCl were used instead of NaCl (not shown). The simplest assumption is that it is due to the Cl^- . However, in this case it should be driven against its concentration gradient.

It is known that an initial large positive membrane potential in LIS is quite stable and does not dissipate during at least 100 s of exposure [10,19]. This indicates that the intracellular Cl^- concentration is almost constant and the loss of Cl^- via Cl^-/OH^- exchange is compensated for by a decrease in cell volume [20]. If the external Cl^- concentration is subsequently elevated up to 20 mM, the potential falls, but the Cl^- gradient is still directed outward, assuming the intracellular Cl^- concentration to be about 80-100 mM [20]. Thus, it is hardly correct to suggest that Cl^-

loading is mediated by some kind of conductive channel-like transport system for this anion (e.g. NSVDC). The operation of the $\text{K}^+(\text{Na}^+)/\text{H}^+$ exchange will also cause cell shrinkage due to obligate water efflux, i.e. the opposite effect.

In the attempt to explain this phenomenon, we used Lew's proposed integrated model of volume regulation [20]. The model predicts monotonic cell shrinkage under isosmotic conditions, if extracellular chloride concentration is reduced; however the cells begin to shrink and demonstrate MR only if the chloride concentration is less than 2-3 mM. The model does not include a special route for bicarbonate transport [20] and cannot explain any influence of the bicarbonate and acetazolamide. Also, a more direct comparison of the experimental results with the model is impossible because the model cannot be used in buffer-free media, the main condition of the present experiments. We conclude that our attempts to explain the results using model simulations were unsuccessful.

From the point of view of the results shown in Fig. 5 and reported elsewhere [21], demonstrating the shrinking effect of the external bicarbonate, it can be suggested that the re-swelling is due to a reversion of bicarbonate gradient. It now becomes necessary for the outward bicarbonate gradient to be greater than the outward chloride gradient. Under the present experimental conditions, the values of the outward bicarbonate and chloride gradients were estimated to be approximately 10 and 5, respectively. The bicarbonate gradient is greater than the chloride gradient and can potentially drive the movement of the chloride ions inside the cells, causing swelling. This proposal implies the dependence of the re-swelling on the concentration of the external chloride (the more the chloride, the lower the outward chloride gradient, the faster the re-swelling), which is not the case because the rate of re-swelling, in fact, does not depend on the chloride concentration (Figs. 1 and 2). It also predicts the DIDS and acetazolamide inhibition due to the direct involvement of the AE1 and carboanhydrase in $\text{Cl}^-/\text{HCO}_3^-$ exchange [22-25]. However, the data show a lack of DIDS and acetazolamide inhibition of the initial fast sucrose-induced shrinking in the bicarbonate-containing medium (Fig. 5, V3). This correlates with the inability of DIDS to inhibit changes in the intracellular pH shown in [26]. At the same time both DIDS and the acetazolamide inhibit the Cl^- -induced re-swelling in the absence of the bicarbonate. The data suggest that the bicarbonate can utilize a transport route distinct from AE1 or carboanhydrase. This view is consistent with the analysis given in [27], that the CAII does not bind directly to the AE1, and that the bound CAII does not substantially accelerate the HCO_3^- transport.

Finally, all the typical volume and shape responses shown in Fig. 5 are almost identical for normal RBCs and bRBCs. The latter have an intracellular bicarbonate concentration around 6-8 mM, indicating that the magnitude of the outward bicarbonate gradient has no principal bearing on the responses. In summary, these data show that Cl^- -induced re-swelling in LIS cannot be satisfactorily explained by the reversion of OH^- or HCO_3^- gradients being able to drive the Cl^- inside the cells against the concentration gradient. However, the data demonstrate a significant role played by the external bicarbonate ions in shape and volume responses in the LIS, a mechanism which warrants further research.

We hypothesize that the native red blood cell is equipped with mechanisms that can be initially be designated as a volume responsive system (VRS) and a shape responsive system (SRS).

These systems are able to sense and respond to changes within the cell environment, making the cell "active" in this sense. The data presented show that the systems can operate very quickly at some circumstances (e.g. in response to bicarbonate, Fig. 5) which make them experimentally difficult to observe. Therefore, the final cell volume as well as shape is not constant at a given medium osmolarity and composition, and most often, has little in common with the intrinsic osmotic behavior of the red blood cells. This is confirmed by the data shown in Fig. 5 (V1), where the cells have a relative volume ranging from 0.5 to 0.68, whereas the predicted volume is 0.74 (476 mosm). The data showing the diverse changes in the volume and shape of the cells in LIS, induced by small amounts of organic anions (less than 1 mM), also concur with this suggestion. This demonstrates a critical dependence of these parameters on the composition of the medium (unpublished data). It is our hope that a better understanding of the functional principles of VRS and SRS is considered serious enough to warrant future research.

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Appendix to the article

Relationship between absorbance and shape index during MR and recalculating the procedure to convert raw absorbance and SI data into relative volume changes.

Absorbance changes of red cell suspension have two major components – one responsible to volume changes ΔA_v and other to shape changes ΔA_{sh} .

$$\Delta A = \Delta A_v + \Delta A_{sh} \quad (A1)$$

Osmotic behavior of red cells is usually correspond to that of nonideal osmometer and can be described by modified Van't Hoff equation in terms of cell volume as follows:

$$\frac{V_c}{V_o} = 1 + R \cdot W \left(\frac{C_o}{C} - 1 \right) \quad (A2)$$

where V_o and C_o are initial cell volume and osmolarity of the medium, W – volume fraction of cell water in normal isosmotic conditions (0.7 [20]) and R – an empirical coefficient to fit to experimental results [28]. For ideal perfect osmometer $R=1$, for human erythrocyte the magnitude for R is expected to be $\sim 0.5-0.7$ [29-31]. Assuming the relation (A2) is valid, changes in absorbance due to only volume changes of cells at given concentration can be written in the form:

$$A = A_o + b_v \left(1 - \frac{C_o}{C} \right) \quad (A3)$$

where b_v is an empirical coefficient of proportionality converting the volume changes into absorbance changes. The validity of expression (A3) was verified in SSS for DIDS treated cells, for cells in the presence of DIDS at different phases of MR and for native cells after addition of aluminum chloride. The reason to use DIDS and aluminum is that both are able to block the shape changes but do not influence osmotically induced volume changes in sucrose. Fig. A1 shows the dependence of absorbance on inverse relative osmolarity in SSS after stepwise addition of NaCl.

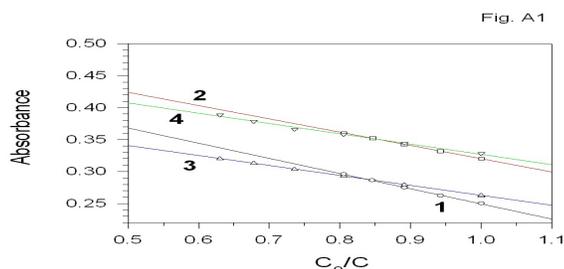


Figure A1. Dependence of absorbance on the inverse osmolarity of the external SSS media changed by the addition of concentrated NaCl at the constant shape of red blood cells. Curve 1: cells fixed by DIDS (2 μ M) at the peak position of MR (~ 20 s after injection of cells into SSS); curve 2: cells fixed by DIDS at terminal phase 3 of MR (~ 150 s); curve 3: cells treated by DIDS as described in material and methods in the presence of 0.025% plasma; curve 4: cells fixed by aluminum (20 μ M) at the peak position of MR. All experimental data are corrected by dilution factor to account for decrease in cell concentration after addition of NaCl. Parameters of linear regression fit of experimental data in the form $y=a+b*x$: curve 1- $a=0.487$, $b=-0.237$, $r^2=0.9989$; curve 2- $a=0.527$, $b=-0.207$, $r^2=0.9998$; curve 3- $a=0.418$, $b=-0.156$, $r^2=0.9998$; curve 4- $a=0.489$, $b=-0.160$, $r^2=0.9930$.

In all cases the dependencies are strikingly linear confirming the validity of expression (A3) with high precision. Slope of this relationship is slightly different for samples used that reflect possible variations in either osmotic behavior or optical properties of the cells after different treatments. We conclude, therefore, that empirically, relationship between absorbance and inverse osmolarity of constant cell shape can be described by the formula (A3).

When the cells change their shape from discoid to more spherical form absorbance is increased. As has been shown in [8] relationship between absorbance and SI during MR is linear with the slope depending on the phase of process. Slope has a larger value in phase 3 where shape and volume changes proceed in one direction (sphering and shrinkage) and lower in phase 2 where shrinking is accompanied with the restoration of discoid shape. Therefore, the component of absorbance responsible for shape changes of constant volume can be written in the form:

$$A_{sh} = A_o + b_{sh}(SI_o - SI) \quad (A4)$$

where b_{sh} is a coefficient which determines relative contribution of shape to the total absorbance value of cell suspension and SI_o is initial SI. It is tempting to speculate that in SSS the value of b_{sh} which correspond to isovolumetric sphering lies somewhere between b_1 for phase 2 ($b_1=0.022$) and phase 3 ($b_1=0.051$). The mean from these values gives $b_{sh}=0.0365$. Other approach to estimate the value of b_{sh} is a direct measuring of relative changes in absorbance and SI during shape transformation of constant volume. Fig. A2 shows a set of measurements performed in different experiments and conditions where the shape transformation is expected to proceed with no or limited volume changes. Although the scattering in the data is rather significant, linear regression fit returns the value of $b_{sh}=0.0354$, i.e. very close to aforementioned mean value.

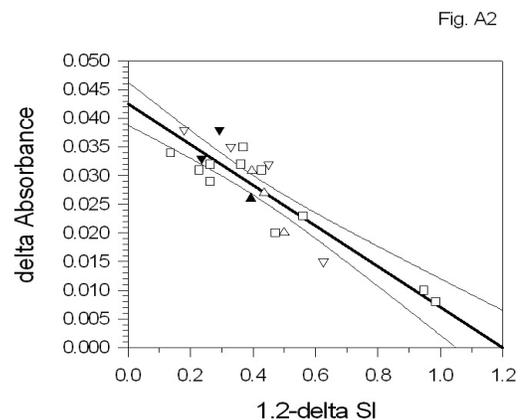


Figure A2. Dependence of absolute absorbance changes (ΔA) on changes in SI (ΔSI) during shape transformation of constant volume. Each point corresponds to a separate experiment under different conditions and using different blood samples. Experiments included measurements of shape changes induced by 20 μ M DIDS in SSS containing 10 mM NaCl (\square) or in HBS (Δ), or in SSS containing 20 mM NaCl and BzA (0.4 μ g/ml) (Δ), or induced by 0.2 μ g/ml of BzA in SSS containing 20 mM NaCl and 0.4 μ g/ml BzA (τ). Absorbance changes (ΔA) and changes in SI (ΔSI) were determined as a difference between an initial stable value before reagents addition and a new stable value after the addition of reagents. Parameters of linear regression fit of experimental data in the form $y=a+b*x$ (bold line) are: $a=0.0424$, $b=-0.0354$, $r^2=0.8147$; Thin lines show 95% confidence interval for these data.

Taking in mind two components of absorbance, the total absorbance changes contributed to both volume and shape can be written in terms of osmolarity as follows:

$$A = A_o + b_v \left(1 - \frac{C_o}{C}\right) + b_{sh}(SI_o - SI) \quad (A5)$$

or using formula (A2)

$$A = A_o + \frac{b_v}{R \cdot W} \left(1 - \frac{V_c}{V_o}\right) + b_{sh}(SI_o - SI) \quad (A6)$$

from which we get the final expression for relative volume changes during simultaneous volume and shape changes of cells in suspension based on measuring of raw absorbance and SI data:

$$\frac{V_c}{V_o} = 1 - \frac{R \cdot W}{b_v} (A - A_o - b_{sh}(SI_o - SI)) \quad (A7)$$

At reference state, that is in the normal isotonic physiological saline at normal cell volume and discoid shape, it is assumed that $A_0=0.3$ and $SI_0=1$ and $V_c/V_0=V_{rel}=1$. If the cells change only their volume and not a shape this will result in an increase in absorbance corresponding to decrease in cell volume and vice versa. Fig. A3 shows theoretical dependence of relative cell volume on absorbance when b_v is varied. Lowering this parameter leads to an increase in relative volume changes in response to the same absorbance variations (Fig. A3A) which is maximal for the cell with $R=1$ and reduces if R value is decreased (Fig. A3B).

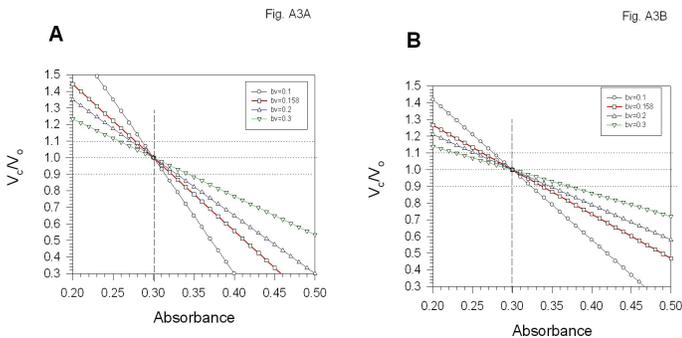


Fig. A3A

Fig. A3B

Fig. A3C

Figure A3. Dependence of the calculated relative cell volume on absorbance (A, B) at different values of parameter b_v , shown in the legends for values $R=1$ (A) and 0.6 (B), and on parameter R (C) at different values of absorbance, shown near the lines. Calculations are done assuming the shape of the cells is constant.

For the lowest experimentally defined coefficient $b_v=0.158$ 10% volume reduction is achieved when absorbance increases by 0.02 AU when $R=1$ and by 0.04 AU when $R=0.6$. Actually, the volume sensitivity directly depends on the ratio R/b_v , however because b_v is determined from the optical measurements and R from direct volume ones [29,32] they should be considered as different parameters where R is a parameter which determines an ability of cell to undergo volume changes in anisotonic media. Fig. A3C shows that volume deflections linearly depend on R value and under maximal possible absorbance changes during MR (~ 0.08 AU) can account for 35% of volume reduction if $R=1$ but only 20% if $R=0.6$. In this estimate, it is suggested that all absorbance changes are attributed solely to volume, however since shape changes also contribute to absorbance, clear that this is an upper estimate and resulting volume changes will be lower. Nevertheless, all these simulations show that absorbance deflection in our case is rather sensitive to change in cell volume. An important question is at what extent the precision of recalculating volume changes depends on the choice of initial reference parameters A_0 and SI_0 ? In physiological salt saline there is not a problem because A_0 and SI_0 are usually stable and directly measured. Even if SI_0 is different from unity as for example for old red cells, both A_0 and SI_0 can be included in formula (A7) for calculation of subsequent volume dynamics. In sucrose solution the situation is more complicated since the initial value of absorbance is significantly lower than in HBS, usually in the range 0.24-0.27 instead of 0.3 and, in addition, the cells immediately change their shape upon introducing them in this solution [8,14]. Therefore, we do not know exact values of A_0 and SI_0 that correspond to initial isotonic volume and discoid shape of the cells at this new environment. Fig. A4 illustrates how volume dynamics during standard MR is affected by the choice of initial parameters A_0 and SI_0 . These data show critical

Fig. A4

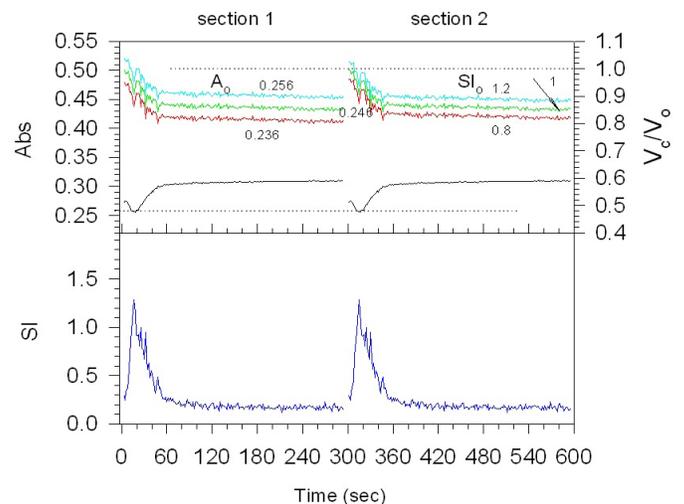


Figure A4. Dependence of absorbance, SI and calculated relative cell volume on time during standard MR in SSS and the effect of the initial parameters A_0 and SI_0 on the time course of relative cell volume changes. In the top graph, the upper curves correspond to the relative volume whereas the lower ones correspond to absorbance changes. Section 1: initial parameters of A_0 are shown near the curves, $SI_0=1.2$; section 2: initial parameters of SI_0 are shown near the curves, $A_0=0.246$.

dependence of absolute values of calculated relative volume on the initial values of A_0 and SI_0 . Increase in both parameters causes an uniform upward shift of the curves whereas a decrease causes inverse downward shift of the same amplitude. Importantly, the relative error in assessment of A_0 (%) is directly converted in the same error of V_{rel} (%). In contrast, 20% error in SI_0 produced only about 3% difference in V_{rel} . One may conclude, that in order to get quantitatively precision V_{rel} data, the initial value of A_0 should be also determined with the same accuracy. This can be achieved in separate experiments where the cells initially do not change the shape, as for example in the presence of NaCl, at low pH or calcium in a sucrose solution [8,14] or adjust A_0 so that initial V_{rel} would not substantially be different from unity, as in Fig. A4

implies that at the first moment the volume of cells is equal to isotonic volume in the physiological saline. Finally, an estimate of contribution of b_{sh} in the final value of V_{rel} shows the negligible influence of this parameter producing not more than 2.5% error when varying b_{sh} within $-10 - +10\%$ of initial value of 0.036. Therefore, by fixing this parameter in all experiments constant, it could be possible only a small systematic error in absolute value of V_{rel} that has no principal meaning in assessing the time-dependent relative changes in cell volume. Based on this consideration, we will use the following fixed model parameters in recalculating procedure: $R=1$, an ideal linear perfect osmometer; $W=0.7$, the fraction of water in the cell; $b_v=0.158$, to provide the maximal sensitivity in the determination of volume changes; $b_{sh}=0.036$.
