БИОЛОГИЧЕСКИЕ МЕМБРАНЫ, 2003, том 20, № 2, с. 169–177

УДК 576.3

MAINTENANCE OF A CONSTANT AREA-TO-VOLUME RATIO IN DENSITY-FRACTIONATED HUMAN ERYTHROCYTES

© 2003 г. I. L. Lisovskaya, J. M. Rozenberg, E. E. Yakovenko, F. I. Ataullakhanov

National Research Center for Hematology, Russian Academy of Medical Sciences, Novozykovskii pr. 4a, 125167 Russia, Moscow, Phone + 7 95 212 5531; e-mail irene_l@mail.ru

Received 25.04.2002

It is well known that senescent erythrocytes have smaller sizes, higher density, and smaller surface area than younger ones. The area-to-volume ratio is one of the main factors that determine erythrocyte deformability. However, the mechanisms ensuring the correlation between the volume (V_0) and surface area (A) of a particular erythrocyte remains unclear. Besides, the exact relationships between the membrane area and cell volume are under discussion. Some authors believe that, during *in vivo* aging, erythrocytes exhibit constant value of the membrane area-to-volume ratio ($R = A/V_0$). Others argue that it is the sphericity ($S = 4.84V_0^{2/3}/A$) that does not change as cells are aging. To test these two hypotheses, we have used the osmofiltration method. Numerical analysis shows that measurements of erythrocyte filterability vs. medium osmolality for cell fractions of various density make it possible to determine which of the relationships, R = const or S = const, is closer to reality. The experimental data show that it is the area-to-volume ratio (R) that remains nearly constant in circulating erythrocytes. We suppose that as yet unknown regulatory mechanisms exist in human erythrocytes and/or in the microcirculation system as a whole, that maintain the erythrocyte area-to-volume ratio constant during the erythrocyte life span.

The relatively long life span of human erythrocytes in the circulation (about 120 days) implies their high resistance to stresses. However, over the life of the cell it's properties experience progressive changes that eventually cause the removal of the senescent cell from the circulation [1–6]. The vast majority of studies in the field of erythrocyte aging used density fractionation to produce populations of light (young) and dense (aged) erythrocytes. [3, 6, 7]. Comparing light and dense cell populations, one can assess the changes the cells are subject to as they age.

It is well known that frequency distributions of volume (V_0) and surface area (A) of circulating erythrocytes of healthy donors, as well as the distribution of intracellular hemoglobin concentration (HC), are approximately normal (Gaussian) [5–9]. The ability of erythrocytes of different ages and sizes to adequately carry out their oxygen-transport function while moving through the blood vessels, implies some limitations on their geometric parameters. As early as in 1960s, parallel measurements of A and V_0 of individual erythrocytes in the microphotographs lead Canham to conclude that these parameters were correlated [10, 11]. This conclusion was further confirmed by numerous direct measurements (mainly based on the micropipette technique) of A and V_0 of individual erythrocytes.

However, a controversy exists among researchers on the issue of the nature of this correlation. According to some authors, erythrocytes exhibit constant value of the surface area-to-volume ratio: $R = A/V_0 = \text{const}$ [7, 8, 10]. At the same time, Waugh et al. [5] argue that the constant that couples A and V_0 is not the area-to volume ratio R, but a so-called sphericity S, defined as the inverted ratio of an erythrocyte surface area to the area of a sphere of the same volume $S = A_{sph}/A = 4.84V_0^{2/3}/A$.

The surface area-to-volume ratio is a significant factor that determines deformability of erythrocytes [9, 11, 12] and eventually their ability to pass through the capillar system and deliver oxygen. It is therefore important to find out, which of these two relationships: R = const or S = const, describes the real situation more accurately. To examine this issue, we make use of a fact that smaller cells have on the average higher density [5, 6]. The whole population of normal erythrocytes can be split into fractions of large and small cells and the parameters of interest can be evaluated for each fraction.

In order to determine mean cells surface area, we employed the osmofiltration method developed in our laboratory earlier [13, 14]. The idea of the method is as follows. The known way to change erythrocyte volume without changing its surface area is to alter the medium osmolality [7, 13]. As the osmolality decreases, the volume (but not the surface area) of the erythrocyte increases. At some critical volume (V_{cr}), the cell loses the ability to pass through a filter with pores of a given diameter and length, that is, the erythrocyte "filterability" reaches zero. For a given filter, the critical volume of the erythrocyte depends on surface area only [13]. By measuring the dependence of erythrocyte filterability on osmolality, one can determine the critical value of osmolality (u_{cr}) related to the cell critical volume V_{cr}, cell surface area and the cell initial ("isotonic") volume V_0. As it will be shown below, the dependence of filter-

ability on the medium osmolality for erythrocyte fractions of various densities allows determining which of the relationships involved, R = const or S = const, is closer to reality. The experimental results provide evidence that it is the area-to-volume ratio that remains nearly constant in circulating erythrocytes.

NUMERICAL MODEL FOR THE DISTRIBUTIONS OF THE CRITICAL OSMOLALITY u_{cr} IN LIGHT AND DENSE ERYTHROCYTE FRACTIONS. A GROUND FOR EXPERIMENTAL APPROACH

It has been previously shown in [13] that the critical volume of an erythrocyte can be calculated if its surface area and the parameters of a filter pore are known:

$$V_{cr} = \frac{dS}{4} - \frac{\pi d^3}{12} \quad \text{if} \quad S < \pi d^2 + \pi dl, \quad \text{and}$$

$$V_{cr} = \frac{\pi d^2 l}{4} + \frac{(S-\pi dl)^2}{3C} - \frac{\pi d^2 (S-\pi dl)}{6C} + \frac{d^2 C}{24} \quad (1)$$

$$\text{if} \quad S > \pi d^2 + \pi dl,$$

where d is the mean pore diameter (3.2 μm), l is the thickness of the filter (7 μm), and

$$C = \left(8\pi\left(S - \pi d\left(l + \frac{d}{2}\right)\right)\right)^{0.5}.$$

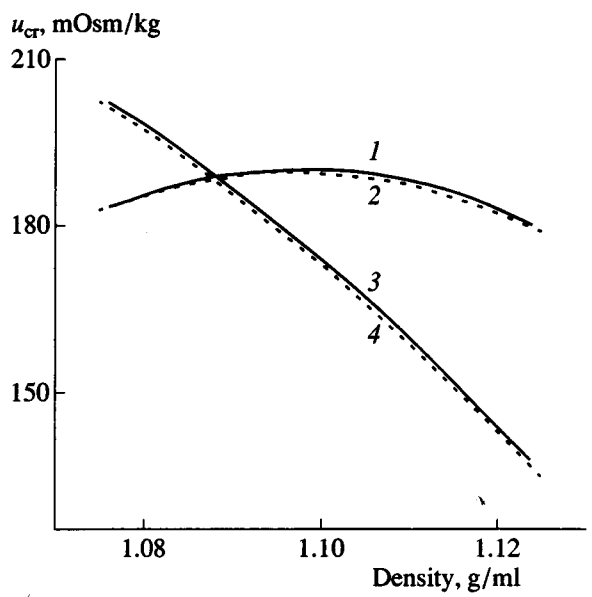

Fig. 1. Critical osmolality u_{cr} vs. erythrocyte density. The curves are calculated according to the model for the density distribution width CV of 0 (solid lines *1*, *3*) and 1% (dotted lines *2*, *4*). Other parameters used in calculations are as follows: (*1*) H = 30 pg/cell, $R = A/V_0$ = 1.68 μm^{-1}; (*2*) H_c = 30 pg/cell, CV = 12.8%, $R = A/V_0$ = 1.68 μm^{-1}; (*3*).

H = 30 pg/cell, $S = 4.84 V_0^{2/3}/A$ = 0.64; (4) H_c = 30 pg/cell, CV = 12.8%, $S = 4.84 V_0^{2/3}/A$ = 0.64.

To alter the cell volume while keeping the rest parameters unchanged we vary the medium osmolality. It is known that erythrocyte volume is inversely proportional to the osmolality:

$$V = V_{min} + f/u, \quad (2)$$

where V_{min} is a minimal volume of a cell at infinite osmolality

$$V_{min} = H/\alpha, \quad (3)$$

where α = 69.44 g/dl, H – hemoglobin content in a cell and f is a coefficient of osmotic sensitivity. A critical volume V_{cr} would be related to critical osmolality u_{cr} that, taking (2), into account, can be evaluated as

$$u_{cr} = f/(V_{cr} - V_{min}). \quad (3a)$$

If an erythrocyte has a volume V_0, density , and contains H pg/cell of hemoglobin under isoosmotic conditions (i.e., u_0 = 300 mOsm/kg), then

$$V_0 = V_{min} + f/u_0 \quad (3b)$$

with the hemoglobin content being linked with a cell volume and density as follows [5]:

$$H(\rho_h - 1) = V_0 \rho_h (\rho - 1), \quad (4)$$

where ρ_h is specific density of hemoglobin (1.36 g/ml) and ρ – cell density.

From (3a), (3b) and (4) the dependence of an osmotic sensitivity (f) and critical osmolality (u_{cr}) on cell density (ρ) can be calculated:

$$f = u_0(V_0 - V_{min}) = u_0\{H(\rho_h - 1)/(\rho_h(\rho - 1)) - H/\alpha\} = \\ = u_0 H\{(\rho_h - 1)/(\rho_h(\rho - 1)) - 1/\alpha\} \quad (5)$$

and

$$u_{cr} = f/(V_{cr} - H/\alpha) = \\ = u_0\{(\rho_h - 1)/(\rho_h(\rho - 1)) - 1/\alpha\}/(V_{cr}/H - 1/\alpha). \quad (6)$$

To obtain critical volume (V_{cr}), cell surface area has to be detected. Using Eqn. (4), it is possible to calculate how cell surface area varies with density and hemoglobin content at either constant area-to-volume ratio (R = = const) (Eq. 7), or constant sphericity (S = const) (Eq. 8):

$$A' = RV_0 = RH(\rho_h - 1)/(\rho_h(\rho - 1)), \quad (7)$$

$$A'' = 4.84 V_0^{2/3}/S = \\ = (4.84/S)\{H(\rho_h - 1)/(\rho_h(\rho - 1))\}^{2/3}. \quad (8)$$

Substituting (7) or (8) into (1), we will get the critical volume V_{cr}. Further substituting V_{cr} into Eqn (6) gives critical osmolality as a function of cell density. These dependencies calculated for a fixed value of H are presented in Fig. 1 by curves *1* and *3*. Curve *1* was obtained assuming constant area-to-volume ratio, whereas curve *3* – assuming constant sphericity.

For the sake of simplicity, we supposed that erythrocyte hemoglobin content was constant. However, taking into account the hemoglobin content distribution, as well as erythrocyte density distribution within each fraction (see Appendix), gave virtually similar dependencies of the critical osmolality on density (Fig. 1, curves *2* and *4*). The two alternative hypotheses concerning the relation between A and V_0 resulted in two qualitatively different dependencies of the critical osmolality on density. At a constant area-to-volume ratio, critical osmolality weakly grows with the density at small values of ρ, with some decrease at higher values. In contrast, if sphericity is constant, u_{cr} quickly drops as the density increases.

The theoretical considerations developed above suggest that the measurement of the critical osmolality for density-separated erythrocyte fractions can help to determine relationships between erythrocyte volume and surface area. If the critical osmolality weakly depends on density, then the area-to-volume ratio is close to constant. If the critical osmolality u_{cr} considerably decreases with growing density – then it is the sphericity that is kept constant.

EXPERIMENTAL

Erythrocytes and solutions. Erythrocytes from blood (anticoagulated with acid citrate decstrose) of healthy adults were isolated by centrifugation, supernatant was saved (and further used for the density separation, see below). Subsequently erythrocytes were washed twice in HEPES-buffered saline (HBS-1, 145 mM NaCl, 5 mM KCl, and 10 mM HEPES, pH 7.4, 300 mOsm/kg). The cells were washed in HBS-2 (20 mM HEPES, 5 mM KCl, 1 mM $MgCl_2$, 1 mM NaH_2PO_4, 128 mM NaCl, 5 mM glucose, and albumin 0.2%, pH 7.4, 300 mOsm/kg) and resuspended in this solution at a hematocrit of about 30%. After each centrifugation the buffy coat was carefully removed by aspiration. All measurements were performed within 24 h from the moment of the blood collection and within 5 h after the preparation of washed erythrocytes.

The osmolality of each solution was determined with an OMKA 1 Ts–01 cryoscopic osmometer (Russia). All solutions were filtered through 0.22-μm Millipore filters before use.

Density separation. The method described by Linderkamp and Meiselman [7] with some modifications was used for density separation. Briefly, the samples of washed erythrocytes were sedimented and resuspended in autological plasma (saved during the erythrocyte isolation) at the hematocrit of about 80%. The suspension was placed into 47-mm long plastic tubes (inner diameter, 4 mm) and centrifuged at 5400 *g* for 40 min. After centrifugation plasma was discarded and each tube was cut at 1 mm below top border of cells and 40 μl of the lightest erythrocytes were carefully removed and resuspended in 10 ml of HBS-2 (top cells). The tube was cut again close to its lower end and 40 μl of the densest erythrocytes was removed and placed into HBS-2 (bottom cells). Top and bottom fractions obtained in such a way contained about 8% of the lightest and about 8% of the densest erythrocytes. We will hereafter refer to the erythrocyte fractions thus obtained as to "top" and "bottom" fractions. Erythrocyte density distributions in both fractions were evaluated using phthalate method of Danon and Marikovsky [15].

Another method for density separation employed one-step percoll-hypaque density gradient. A stock percoll-hypaque density gradient solution (PHS) was prepared according to [16]: 9 ml of Percoll, 2 ml of Na-diatrizoate solution (1 g Na-diatrizoate ("Sigma") in 2 ml H_2O), 6 mg of Penicillin (1585 units/mg), 13 mg of Streptomycin. The pH of stock density gradient solution was adjusted at 7.4. Then stock PHS was diluted with HBS–1 to get PHSs with different densities.

In order to separate the light erythrocyte fraction, washed cells were purified using α-cellulose microcrystalline cellulose columns [17] or Imugard cotton [18] to eliminate leukocyte and platelet contamination, and then resuspended at hematocrit of 10% in HBS-2. Percoll-hypaque solution in the amounts of 200 μl which density varied within 1.075–1.095 g/ml was placed at the bottom of flexible plastic tubes with the inner diameter of 5 mm and length of 93 mm. Then about 1600 μl of 10% erythrocyte suspension was carefully laid over. The sample was centrifuged for 7 min at 1800 *g*.

To separate dense erythrocyte fraction, 200 μl of PHS with density of 1.100–1.113 was also placed into the tubes, with washed erythrocytes resuspended in the autological plasma at the hematocrit of about 80% being laid over and the whole sample being centrifuged for 12 min at 1800 *g*.

Once the centrifuging was completed, the lightest or the densest cells, separated from the rest of erythrocytes by a transparent PHS band, were collected into one-ml syringe by piercing a plastic tube. We will refer hereafter to erythrocytes separated by means of PHS, as to "light" and "dense" ones. All density-separated fractions contained no more than 0.1 leucocytes and 5 platelets per 1000 erythrocytes.

Filtration measurements using a kinetic filtrometer. We have developed earlier a kinetic filtrometer to continuously record the filtration rate of erythrocyte suspension [19]. Filtration was performed under constant pressure of 245 Pa through a nickel mesh filter with uniform pores (diameter, 3.1 μm; length, 3.5 μm). (TSUCASA SOKKEEN CO. LTD., Japan) [20]. The volume of erythrocyte suspension passing through the filter was recorded as a function of time by a linear optoelectronic device. Each time before the filtration of cell suspension, a flow rate of the suspending medium (w_b) was determined. The same filter was used in all experiments. Mean relative rate of the passage through a pore of an individual erythrocyte from the major subpopulation ($w(u) = w_e/w_b$ where w_e is mean rate of the

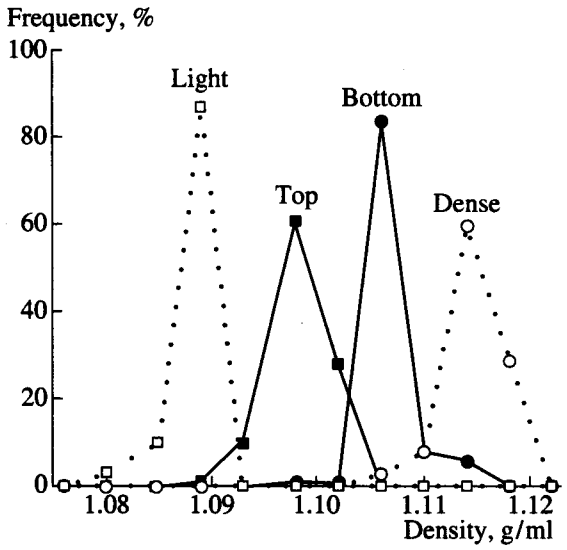

Fig. 2. Density distributions of erythrocyte fractions isolated by the modified Linderkamp method (solid lines) or by centrifugation in a discontinuous one-step percoll-hypaque gradient (dotted lines).

passage through a pore of an erythrocyte) was determined from the kinetic filtration curves recorded by the filtrometer at various osmolality values [19]. The u_{cr} value was determined by extrapolating the $w(u)$ curve to $w = 0$ using a linear regression procedure.

Filtration measurements by a Hanss-type hemorheometer. Density-separated erythrocytes were suspended in HBS-2 to a hematocrit of 30% and used within 2 h after preparation. Before measurements, the suspensions were diluted to a hematocrit of 0.1 or 1% with HBS-1 containing NaCl at concentrations that provided the desired value of osmolality u in the final suspensions.

Relative filtration rates of the erythrocyte suspensions were measured using the device, which is our modification of Hanss' hemorheometer (Russian Patent #2052194). The filterability of erythrocyte suspensions was measured as t_b/t_s ratio where t_s and t_b are the times it took 250 μl of HBS-1 with and without cells, respectively, to pass through a membrane filter at 22 ± 1° C and pressure of 6 cm H_2O.

Our modification of Hanss' hemorheometer allowed measuring t_b and t_s to an accuracy of 0.1 s. The instrument was not thermostated, but sample temperature variations were insignificant because both buffer and suspension were kept at 22 ± 0.2°C in a water bath throughout the experiment. Membrane filters were made of a polyethylene terephthalate film (thickness, 7 μm; mean pore diameter, 3.2 μm [13, 14]; Joint Institute for Nuclear Research, Dubna, Moscow oblast, Russia).

All measurements were performed in triplicate at 22 ± 1°C.

The distributions of u_{cr} for the density-separated erythrocytes were assessed by previously proposed osmofiltration method [21]. Briefly, the relative flow rate t_b/t_s of erythrocyte suspension containing cells with different u_{cr} through a filter gradually decreases with decreasing medium osmolality. This rate becomes zero at some $u = \Omega$, when the number of non-filterable cells in the applied sample approaches the number of pores in filter. Extrapolating the descending portion of the curve t_b/t_s vs. u to lower osmolalities by using a linear regression procedure, we estimated Ω. Evidently, the critical osmolality value Ω depends on hematocrit. With 0.1% suspension, filtering stopped at $u = \Omega(0.1\%)$ when about 30% of cells in the suspension lost the ability of passing through pores, whereas 1% suspension stopped filtering at a greater osmolality $u = \Omega(1\%)$, when 3% of the cells become non-filterable. Assuming that the distribution of u_{cr} is normal, one can evaluate standart deviation (σ) and center position (M) from the relationships $[\Omega(0.1\%) - M] = 0.5\sigma$, and $[\Omega(1\%) - M] = 1.9\sigma$.

$$\sigma = [\Omega(1\%) - \Omega(0.1\%)]/1.4,$$

$$M = \Omega(0.1\%) - 0.5\sigma. \text{ [21].}$$

Table 1. Mean density values of top and bottom fractions of density-separated erythrocytes ($n = 18$)

Density, (g/ml)		Δ, %
Top	Bottom	
1.095 ± 0.006	1.107 ± 0.005	12.4 ± 0.4

RESULTS

The typical density distributions of erythrocytes isolated by the modified Linderkamp method (solid lines) and by the centrifugation in one-step percoll-hypaque gradient, (dotted lines) are presented in Fig 2.

The light and dense cell fractions obtained by one-step percoll-hypaque gradient contained 5 and 2% of all cells, respectively. The densities of the light and dense cells differed by more than 20%. As the amount of cells isolated by this method is rather small, the major part of measurements was performed on erythrocytes separated by the modified Linderkamp technique. Mean densities of top and bottom erythrocytes obtained by the modified Linderkamp method from eighteen healthy donors differ by more than 10% (Table 1).

Fig. 3 presents typical kinetic filtration curves (relative flow rates, vs. time) for top (*1*, *3*) and bottom (*2*, *4*) erythrocyte fractions, at two different osmolality values of the medium. In each fraction, there is a distribution of the relative rate of cell passage through a pore (w) Extrapolation of a kinetic filtration curve to zero moment of time makes it possible to determine the value w corresponding to its distribution center (mean relative rate of erythrocyte passage through a pore) [19].

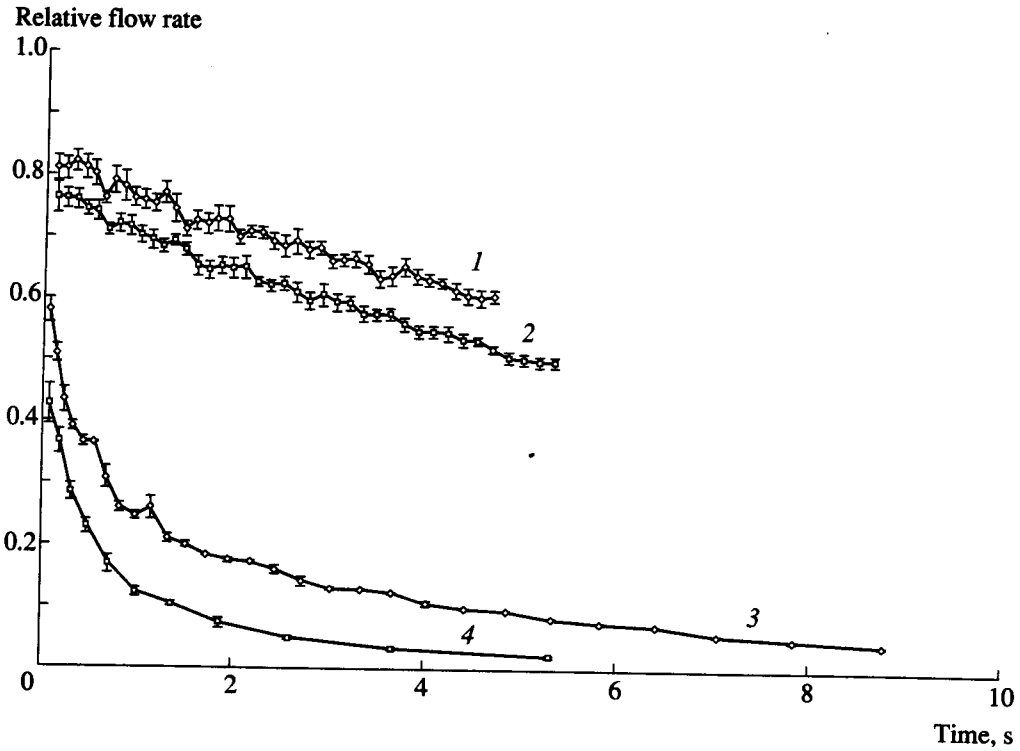

Fig. 3. Time course of the relative flow rate (see text for definitions). Curves *1*, *3* – top erythrocyte fraction and *2*, *4* – bottom erythrocyte fractions; curves *1*, *2* – osmolality 295, curves *3*, *4* – 228 mOsm/kg. Vertical bars indicate standard error.

Figure 4 shows how mean relative rate of erythrocyte passage through a pore w depends on osmolality for top and bottom cell fractions, respectively. Erythrocytes of the bottom fraction pass through the filter much slower in isotonic and hypertonic media than do the cells of the top fraction under the same conditions.

As the osmolality decreases, the passage of the cells through the filter pores gets slower and stops at about 200 mOsm/kg in the case of the top fraction and at 206 mOsm/kg for the bottom cells. The arrow indicates critical osmolality value calculated after formulae (1)–(8) for a hypothetical cell having volume $V = V_{Bot}$ and sphericity $S = S_{Top}$. The critical osmolality for such a cell is u_{cr} = 193 mOsm/kg, which is significantly lower than that experimentally measured.

As it was earlier shown, the critical osmolality distribution can be estimated by means of the osmofiltration technique using the Hanns-type hemorheometer [14]. Fig 5 shows the results of two typical experiments performed using the Hanss-type hemorheometer in which the relative filtration rates of suspensions of density-separated cells were determined as a function of medium osmolality. In one case erythrocytes were separated by the modified Linderkamp method and 0.1 and 1% suspensions were used for filtration measurements. In the other case erythrocytes were separated by centrifugation in percoll-hypaque gradient. This fractionation technique makes it possible to isolate erythrocytes greatly differing in density (Fig. 2), but the low yield of the method allows using only 0.1 % suspensions for the filtration measurements. As shown in Fig. 5, in the case of 0.1% suspensions, the curves for light and dense cell fractions differ only slightly, and in the case of 1% suspensions, the curves for the top and bottom cells differ to a greater extent: $\Omega(1\%)_{Top}$ = = 200 mOsm/kg, whereas $\Omega(1\%)_{Bott}$ = 210 mOsm/kg.

The mean values of $\Omega(0.1\%)$ and $\Omega(1\%)$ for the top and bottom fractions from nine blood samples are shown in Table 2.

The assumption that density-separated erythrocytes in both fractions obey normal distribution with respect to u_{cr} leads to the following estimates:

$$\sigma_{Top} = [\Omega_{Top}(1\%) - \Omega_{Top}(0.1\%)]/1.4 = 10.1 \text{ mOsm/kg},$$

$$M_{Top} = \Omega_{Top}(0.1\%) - 0.5\sigma_{Top} = 186.2 \text{ mOsm/kg}$$

and

$$\sigma_{Bott} = [\Omega_{Bott}(1\%) - \Omega_{Bott}(0.1\%)]/1.4 = 13.1 \text{ mOsm/kg},$$

$$M_{Bott} = \Omega_{Bott}(0.1\%) - 0.5\sigma_{Bott} = 186.1 \text{ mOsm/kg}.$$

This means that the centers of the u_{cr} distributions almost coincide for the top and bottom fractions, but the dense fraction is more heterogeneous: $\sigma_{Bot} > \sigma_{Top}$.

Note, however, that for the densest erythrocytes (density > 1.110 g/ml) accounting for less than 1% of all cells, the center of the u_{cr} distribution was shifted to

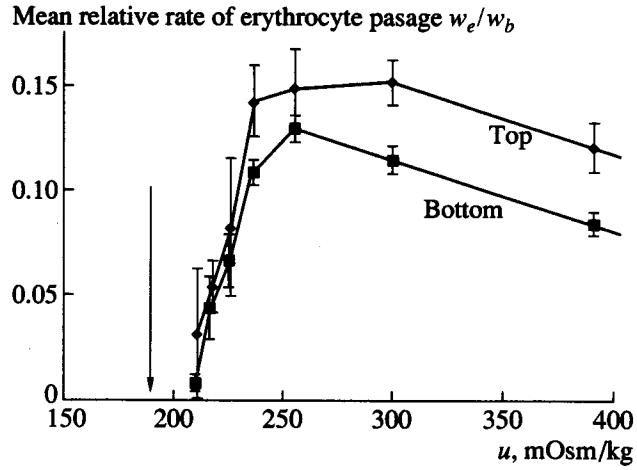

Fig. 4. Mean relative rates of erythrocyte passage through the filter (w_e/w_b) vs. medium osmolality u (n = 4). Diamonds – top erythrocyte fraction, squares – bottom erythrocyte fraction. Arrow indicates the critical osmolality u_{cr} = 193 mOsm/kg as calculated using Eqs. (1)–(8) for a hypothetical cell having volume $V = V_{Bot}$ and sphericity $S = S_{Top}$.

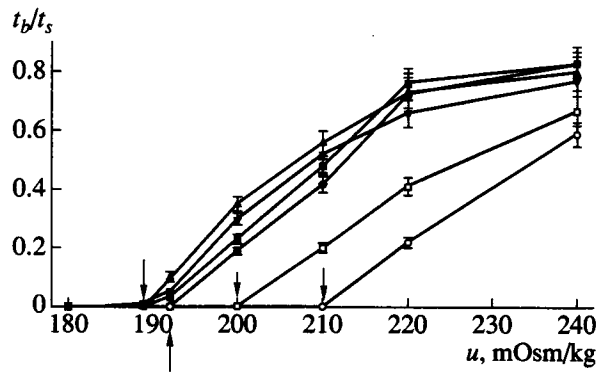

Fig. 5. Relative filtration rate t_b/t_s plotted vs. medium osmolality u for 0.1 and 1% suspensions of density-fractionated erythrocytes. Squares and circles designate top and bottom fractions, respectively, isolated by the modified Linderkamp method; up and down triangles – lightest and densest fractions, respectively, isolated by centrifugation in a discontinuous one-step percoll-hypaque gradient. Filled and open symbols correspond to hematocrite of 0.1 and 1%, respectively. Arrows indicate the Ω value for each curve (See text for definition).

more hypotonic values compared to that of the dense fractions containing 3% of all cells (data not shown). This result suppose that the minor part of the densest cells in population have increased area-to-volume ratio R and is consistent with the data of Waugh who observed the minimal cylindrical diameter (MCD) values in the densest fraction that was accounting for only 0.8% of all cells [6].

Thus the data presented demonstrate that the centers of the u_{cr} distributions for light and dense erythrocyte fractions are quite close, whereas the standard deviation is somewhat greater for dense fractions.

DISCUSSION

Our results show that the osmolality values corresponding to the centers of the u_{cr} distributions of the light (top) erythrocyte fractions are equal or slightly lower than those of dense (bottom) fractions (Figs. 4, 5). Comparison of the experimental curves with the model curves (Fig. 1) obtained under the assumption that constant is either the surface area-to-volume ratio (curves *1, 3*) or sphericity (curves *2, 4*), allows concluding that the first assumption is true. In addition, the deformability (i.e. filtrability) of dense cells in isotonic and hypertonic media is considerably lower than that of the light ones (Fig. 4), presumably because of higher intracellular viscosity.

Large variance of the u_{cr} distributions observed in dense fractions (Table 2) implies that older cells are more heterogeneous than the younger ones.

Note that the maintenance of area-to-volume ratio (but not the sphericity) constant makes the cells of various ages generally more uniform in u_{cr} and in the MCD [6, 10].

Our results are in line with other observations suggesting a decrease of deformability during cells aging [6, 8]. We agree with Linderkamp and Meiselman who concluded that the area-to-volume ratio does not change during aging [7]. However, we disagree with Waugh, who, based on the ektacytometric and micropipette measurements, suggested that it is the sphericity that does not change with cell age [6]. Waugh reported that the densest erythrocytes (that constituted less than 1% of total cells) exhibited significantly increased area-to-volume ratio and decreased MCD, as compared to more light cells. Note that for the densest cell fractions we observed the decrease in u_{cr}, i.e. the increase in the area-to-volume ratio. It is possible that in the minor fraction of the oldest cells approaching the elimination,

Table 2. Mean Ω(0.1%), Ω(1%) and Ω(1%) – Ω(0.1%) values for top and bottom fractions of density-separated erythrocytes (n = 9).

Ω(0.1%)		Ω(1%)		Ω(1%) – Ω(0.1%)	
mOsm/kg					
Top	Bottom	Top	Bottom	Top	Bottom
191.3 ± 9.2	192.6 ± 9.3	205.4 ± 9.6	211.0 ± 2.1	13.7 ± 4.2	18.3 ± 4.9
$P > 0.1$		$P < 0.05$		$P < 0.05$	

the regulatory mechanisms ensuring the maintenance of the area-to-volume ratio throughout life span may be disordered. Gradual deterioration of these mechanisms with age is probably responsible for the wider u_{cr} distribution in dense erythrocytes than that in light cells.

Despite numerous studies devoted to various issues of aging and elimination of erythrocytes, the question of how the correlation between various cellular parameters is implemented remains unsolved. We consider as the most acceptable the hypothesis explaining the erythrocyte aging by the increase of intracellular free calcium concentration (Ca_i) [23]. This increase may result either from an altered permeability of a cellular membrane or from an inhibition of Ca-pumping activity due to mechanical and/or oxydative stresses [1, 24]. The increase of Ca_i level is accompanied by activation of calcium-dependent potassium channels (known as the Gardos effect), so that cells losing potassium ions and water, decrease in volume [25, 26]. A partial loss of surface area may result from exovesiculation, which is also induced by the increase in Ca_i [3, 27].

Note that, by itself, the release of small membrane fragments encapsulating portions of the cytoplasm cannot alter V_0 to any appreciable extent. Obviously, maximum surface losses in in vitro experiments are about 15–20% [28–30]. A similar estimate of the maximum surface loss during aging can be retrieved from experiments on density-separated erythrocytes [6], as well as from the surface area distributions of normal circulating erythrocytes [5, 31]. Assuming the mean diameter of spherical microvesicules to be 0.05–0.3-μm [31], one can obtain an upper limit of the volume loss: it does not exceed 1%.

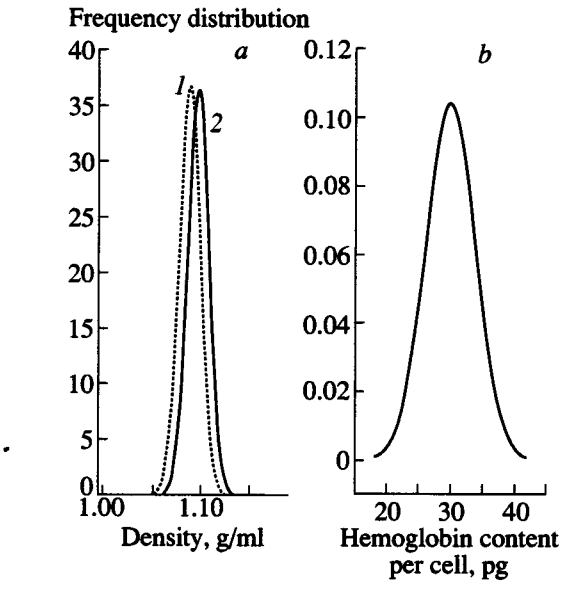

Fig. 6. *a* – Distributions of erythrocyte densities calculated for: (*1*) c = 1.088 g/ml, CV = 1% (dotted line); (*2*) c = = 1.098 g/ml, CV = 1% (solid line). *b* – Distributions of hemoglobin content in erythrocytes calculated for H_c = = 30 pg/cell and CV = 12.8%.

Unambiguous evidence of the influence of the surface area of an erythrocyte on its volume *in vivo* has been obtained in recent works of Waugh [32, 33]. It was shown that after the infusion to mice of labeled autologic erythrocytes with artificially reduced surface area, the mean volume of the labeled circulating erythrocytes decreased.

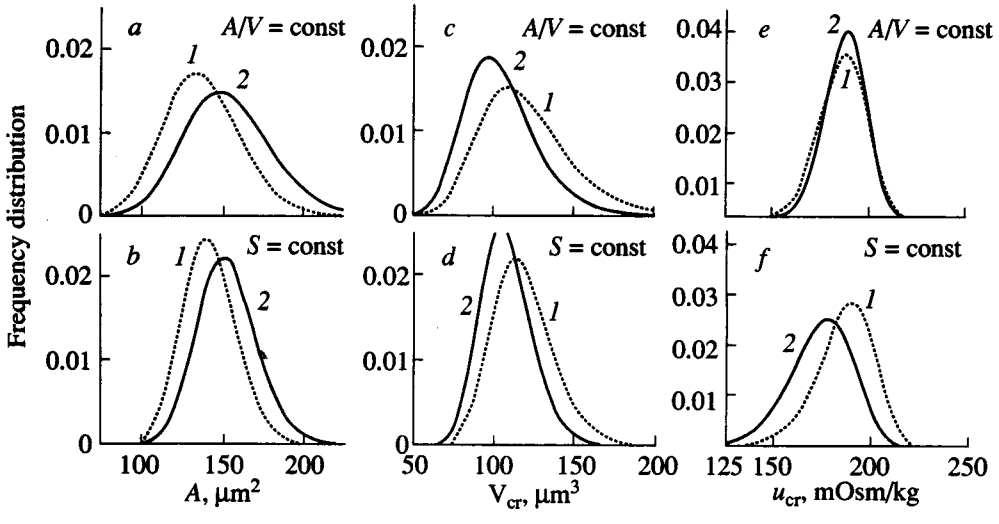

Fig. 7. *a, b* – Surface area distributions calculated for: *a*, A/V_0 = 1.68 μm^{-1}. (*1*) ρ_c = 1.088 g/ml, CV = 1%, dotted line; (*2*) ρ_c = 1.098 g/ml, CV = 1%, solid line; and *b*, S = 0.64. (*1*) ρ_c = 1.088 g/ml, CV = 1%, dotted line; (*2*) ρ_c = 1.098 g/ml, CV = = 1%, solid line. *c, d* – Critical volume (V_{cr}) distributions calculated for: ρ_c, A/V_0 = 1.68 μm^{-1}. (*1*) ρ_c = 1.088 g/ml, CV = 1%, dotted line; (*2*) ρ_c = 1.098 g/ml, CV = 1%, solid line; and *d*, S = 0.64. (*1*) ρ_c = 1.088 g/ml, CV = 1%, dotted line; (*2*) ρ_c = 1.098 g/ml, CV = 1%, solid line. *e, f* – Distributions of critical osmolality (u_{cr}) calculated for: *e*, A/V_0 = 1.68 μm^{-1}. (*1*) ρ_c = 1.088 g/ml, CV = = 1%, dotted line; (*2*) ρ_c = 1.098 g/ml, CV = 1%, solid line; and *f*, S = 0.64. (*1*) ρ_c = 1.088 g/ml, CV = 1%, dotted line; (*2*) ρ_c = 1.098 g/ml, CV = 1%, solid line.

We believe that the area-to-volume ratio is an important regulatory parameter that needs to be maintained at a constant level for the purpose of unifying the rheological properties of erythrocytes at various ages. The maintenance of the area-to-volume ratio constancy is probably provided by a special regulatory system that acts either at the level of an erythrocyte or at the level of circulation as a whole.

APPENDIX

Suppose that for each fraction of erythrocytes with density ρ, a distribution of the hemoglobin content per cell $F(H)$ has a center $(H)_c$ and mean-squared variation $CV = \sigma/(H)$ (Fig. 6b). In [5] it has been shown that the $F(H)$ distribution is approximately similar for erythrocytes of various densities, therefore we will suppose that this distribution is independent of ρ. We also suppose that the whole population is separated into fractions by density, so that each fraction has its particular mean density ρ_c and identical distribution widths CV_p. (Fig. 6a). Using Eqn (4), the volume of a cell with given density ρ and hemoglobin content H, under isoosmotic conditions, can be calculated:

$$V_0 = H(\rho_h - 1)/\rho_h(\rho - 1), \qquad (9)$$

where ρ_h is the hemoglobin specific density (1.36 g/ml).

For a given cell volume, the surface area can be evaluated. The estimation will depend on the relationships between volume and surface area.

1. If the ratio of cell volume (V_0) to surface area (A') is constant ($V_0/A' = R = \text{const}$), then:

$$A' = RV_0 = RH(\rho_h - 1)/(\rho_h(\rho - 1)). \qquad (10)$$

2. If constant is sphericity, i.e., $S = 4.84V_0^{2/3}/A''$ (where A'' is surface area), then:

$$A'' = 4.84V_0^{2/3}/S = \\ = (4.84/S)\{H(\rho_h - 1)/(\rho_h(\rho - 1))\}^{2/3}. \qquad (11)$$

The surface area distributions for each of the two options can be analyzed. Such distributions are presented in Fig. 7*a* for $R = 1.68$ and in Fig. 7*b* – for $S = 0.64$. The two panels show surface area distributions for two fractions: with a mean density of 1.088 g/ml and $CV = 1\%$ (curve *1*) and of 1.098 g/ml and $CV = 1\%$ (curve *2*).

Using (1), one can evaluate the critical volume and obtain the distribution of the critical volumes for a given surface area (Figs. 7*c*, 7*d*). In turn, at a given hemoglobin content and density, a distribution of critical osmolality can be calculated. The assumptions of constant area-to-volume ratio or constant sphericity result in different u_{cr} distributions, as shown in Fig. 7*e* and 7*f*, respectively. We have calculated such distributions for erythrocyte fractions of different densities and obtained the mean critical osmolality value as a function of the fraction mean density (Fig. 1, curves *2*, *4*). The dependencies observed in the experiment are quite similar to those calculated under assumption that all erythrocytes in each fraction possess similar hemoglobin content and have similar density (Fig. 1, curves *1*, *3*).

ACKNOWLEDGMENTS

The authors are grateful to Dr N. Uyesaka (Department of Physiology, Nippon Medical School, Tokyo, Japan) and Dr.T. Kava (TSUCASA SOKKEN CO. LTD, Tokyo, Japan) for the nickel mesh filters.

This work was supported by the Russian Foundation for Basic Research, grant No. 02-04-48155.

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СОХРАНЕНИЕ ПОСТОЯННОГО ОТНОШЕНИЯ ПЛОЩАДИ ПОВЕРХНОСТИ К ОБЪЕМУ В ФРАКЦИОНИРОВАННЫХ ПО ПЛОТНОСТИ ЭРИТРОЦИТАХ ЧЕЛОВЕКА

И. Л. Лисовская, Ю. М.Розенберг, Е. Е. Яковенко, Ф. И. Атауллаханов.

Гематологический научный центр РАМН,
123167 Москва, Новозыковский проезд, 4а; Тел. (095) 212-35-22
электронная почта irenel@mail.ru

В процессе циркуляции (около 120 дней) эритроциты постепенно изменяются (стареют). Более старые клетки имеют меньший объем (V_0), большую плотность и меньшую площадь поверхности (A) по сравнению с молодыми. От этих параметров зависит деформируемость эритроцитов, т.е. их способность проходить по капиллярам микроциркуляции. Все перечисленные параметры при старении клетки изменяются, причем не независимым образом. Например, внутриклеточная вязкость и плотность меняются обратно пропорционально объему клетки. Взаимосвязь изменений площади поверхности и объема эритроцита в процессе старения не столь очевидна. С конца 60-х гг. установлено и в дальнейшем многократно подтверждено существование корреляции между значениями A и V_0. Однако полной ясности в вопросе о характере указанной корреляции нет. Согласно данным одних авторов, объем эритроцита и площадь его поверхности связаны линейной зависимостью: $A/V_0 = R$. Другие авторы полагают, что константой, связывающей A и V_0, является так называемая сферичность $S = 4.84V_0^{2/3}/A$. Чем меньше S, тем больше "избыток" площади поверхности клетки по сравнению с площадью поверхности сферической клетки того же объема. Сохранение постоянной сферичности эритроцитов означало бы, что клетки в процессе старения не меняют свою форму, сохраняя "избыток" площади постоянным. Напротив, постоянство R для всех эритроцитов в популяции означает, что старые клетки имеют меньший избыток поверхности, чем молодые.

В данной работе для решения вопроса о характере соотношения параметров A и V_0 в различных фракциях эритроцитов был использован разработанный нами ранее фильтрационно-осмотический метод. Численный анализ показывает, что измерение зависимости фильтруемости эритроцитов, фракционированных по плотности, от осмотичности среды позволяет определить, какое из соотношений: R = const либо S = const ближе к истине. Результаты проведенных экспериментов показали, что для эритроцитов разной плотности (разного возраста) практически постоянной остается величина R. По-видимому, для циркулирующих эритроцитов отношение $A/V_0 = R$. является важным регуляторным параметром. Мы полагаем, что поддержание постоянства этого отношения обеспечивается регуляторными механизмами, действующими как на уровне клетки, так и на уровне микроциркуляции в целом.