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A Modification of the Filtration Method for Studying the Content of Nonfilterable Cells in Erythrocyte Suspension

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The results of filtration assays provide estimates of the deformability of erythrocytes averaged over the entire suspension. These assays do not distinguish whether the entire population or only its small fraction exhibits abnormal rheological properties. We developed a simple method using a filtrometer to determine the percentage of non-filterable (under given conditions) cells in the erythrocyte suspension. Membrane filters made of a polyethylene terphthalate film had the mean pore diameter of 3.1 μm and the length of cylindrical micropores of 7 μm. The buffer flow rate t_b depends on the number of free pores in a filter. The plot of the number of pores clogged by non-filterable cells versus the total number of erythrocytes passed through the filter had a linear portion whose slope represents the relative content Z of non-filterable cells in the suspension. We determined Z for various medium osmolarities u. These data were used to derive the distribution of erythrocytes in u_{cr}, the value of u at which an erythrocyte cannot pass through a pore of a given filter because of geometric limitations. The distribution maximum corresponded to 190–200 mOsm/kg for erythrocytes from the normal blood. This means that normal erythrocytes have the median values of their surface area and area-to-volume ratio of 155–151 μm^2 and 1.72–1.68 μm^{-1}, respectively. The half-width of the distribution was approximately 30 mOsm/kg. This finding suggests that the normal blood contains a certain fraction of erythrocytes with a decreased area-to-volume ratio. Our results showed that the distribution is altered in various forms of anemia and in ATP-depleted erythrocyte suspensions.

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High deformability of erythrocytes is the necessary condition for realizing their major function: transport of oxygen to tissues. Most techniques for measuring deformability, filtration techniques in particular, characterize

rheological properties of erythrocytes averaged over the entire suspension and do not allow estimation of individual subpopulations, including abnormal ones. At the same time, estimation of the contribution of small subpopulations with modified properties to the blood rheology is the primary task of clinical hemorheology. Most hematological pathologies are accompanied by the appearance of fractions of abnormal erythrocytes in the blood flow, e.g., sickle cells, acanthocytes, spherocytes, ovalocytes, etc. differing by their filtrability from the basic mass of cells [1].

Filtrability of erythrocytes is most often determined from the rate-to-pressure ratio during the passage of cell suspensions through cylindrical pores of membrane filters [2]. For a given cell concentration, the rate of suspension flow under a fixed pressure is known to be determined by a number of parameters: mechanical properties of membranes, intracellular viscosity, and cell geometry [1, 3]. In reality, the situation is more problematic since suspended cells are not identical but distributed in size and other parameters [4]. Neither are identical the pores of a filter [5]. Specifically, Nuclepore filters with a nominal diameter of 3 μm have about 20% of overlapping pores [6]. This makes rather problematic the interpretation of filtrational measurements of deformability. In order to solve this problem, one has to elucidate the relative influences of individual factors on the results of the measurements.

Evidently, under other conditions being equal, the rate of suspension flow through a filter is at each moment dependent on the number of pores accessible (not clogged irreversibly) to erythrocytes. Since the number of pores in a filter is usually small in comparison with the quantity of filtered cells, the availability of even small fractions of non-filtering cells has a considerable effect on the results of filtration measurement [7, 8, 9]. Nowadays, most investigators prefer to use filters with pores 5 μm in diameter and consider plugging the pores as an undesirable side effect [10].

In this work the subject area is namely the clogging of filter pores with erythrocytes during filtration of suspensions.

We proposed earlier a mathematical model describing the dependence of the rate of filtration on the medium osmolality for a homogeneous erythrocyte population [11]. This model relies on the following notions: critical volume of erythrocyte V_{cr} (the minimal cell volume starting from which an erythrocyte of a definite surface area S becomes unable to pass through a pore of a given diameter and length) and critical osmolality of the medium u_{cr} at which an erythrocyte reaches V_{cr} and clogs (plugs) the pore.

This paper describes a modification of the filtration method allowing usage of membrane filters with a mean pore diameter of 3.1 μm for determination of the relative content of non-filtering cells in a suspension as a function of u, i.e., distribution of erythrocytes in u_{cr}, by varying the resuspension medium osmolality u. Specifically, it becomes possible to register the appearance in

suspensions of small subpopulations with modified properties. We derived curves of erythrocyte distribution in u_{cr} in the normal blood, in suspensions of erythrocytes depleted in ATP and in the blood of patients with some forms of anemia.

EXPERIMENTAL

Preparation of erythrocyte suspensions. Erythrocyte suspensions were prepared from human ACD-blood. Erythrocytes were sedimented and washed twice with physiological solution. Each time the supernatant and the buffy coat were removed. The third time erythrocytes were washed with HEPES buffer (145 mM NaCl, 5 mM KCl, 10 mM HEPES, pH 7.4, osmolality 300 mOsm/kg). The cells were resuspended in the same buffer to prepare stock suspensions (hematocrit of 40–50%). To perform filtration measurements, suspensions were diluted with solutions containing 10 mM HEPES and NaCl at concentrations providing the required osmolality. Cell concentrations in suspensions were measured with a Coulter counter hemoanalyzer (France). The mean cell volume was calculated by dividing the hematocrit by the cell concentration.

Treatment of erythrocytes with glutaraldehyde was carried out in the following manner. A 10-ml sample of 1.0% erythrocyte suspension in buffer (osmolality u = 200 mOsm/kg) was mixed with glutaraldehyde to its final concentration of 0.1%, incubated for 15 min at room temperature and washed three times with a 10-fold volume of 0.01 M glycine in the same buffer.

ATP-depleted erythrocytes were prepared by incubating their suspension in isotonic buffer for 20 h at 37°C. Prior to measurements, the cells were again washed three times with isotonic buffer.

After preparation, all solutions were filtered through 0.22 μm Millipore.

Measurement of filtrability index. The index of erythrocyte filtrability was measured using our modification of the Hanss hemorheometer (RF patent No. 2052194). The hydrostatic pressure at a filter was 6 cm H_2O. A sensor was mounted at the top of the column with suspension to record the filtration time of a fixed volume (250 μl) of the cell-free resuspension medium (t_{b0}) or erythrocyte suspension (t_s) to an accuracy of 0.1 s. We used membrane filters made of a polyethylene terphthalate film 7 μm thick and with pores 3.1 μm in diameter (M-3). Nuclepore filters 13 μm thick and with pores 2.4 μm in diameter were also used for comparison.

The pore diameter was measured with the help of a micrometric device on photographs taken with a scanning electron microscope (×2000). Latex beads 2.01 μm in diameter (Coultronics, France) were used for calibration.

Osmolality of solutions was measured using an OMKA 1Ts-01 cryoscopic osmometer (Russia). All measurements were done at room temperature for

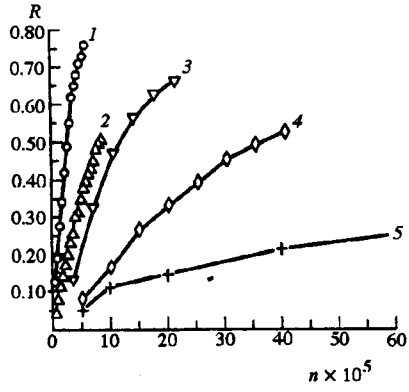

Fig. 1

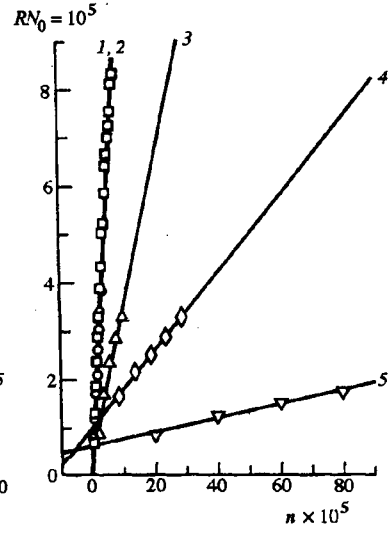

Fig. 2

Figure 1. Dependences of the relative content of clogged pores (R) on the number of filtered erythrocytes (n). *1*, suspension of ervthrocytes treated with glutaraldehyde; *2*, suspension of erythrocytes tre ed with glutaraldehyde, Nuclepore filter; *3*, suspension of intact erythrocytes in isotonic medium containing 17.5% erythrocytes treated with glutaraldehyde; *4*, suspension of intact erythrocytes in isotonic medium containing 7% erythrocytes treated with glutaraldehyde; *5*, suspension of intact erythrocytes in the medium with osmolality $u = 235$ mOsm/kg. Except curve 2, all other curves were derived using M-3 filtres.

Figure 2. Dependences of the number of clogged pores (RN_0) on the number of filtered erythrocytes (n). *1*, suspension of erythrocytes treated with glutaraldehyde ($k = 1.00$); *2*, suspension of erythrocytes treated with glutaraldehyde, Nuclepore filter ($k = 0.98$); *3*, suspension of intact erythrocytes in isotonic medium containing 29% erythrocytes treated with glutaraldehyde ($k = 0.3$); *4*, suspension of intact erythrocytes in the medium with osmolality $u = 231$ mOsm/kg ($k = 0.01$). Except curve 2, all other curves were derived using M-3 filtres.

6 h after washing the erythrocytes. For each filter, t_{b0} was measured by passing the buffer through it, and t_s was determined by passing the erythrocyte suspension with 1% hematocrit through the same filter. The filtrability index was determined as $F = t_{b0}/t_s - t_{b0}$ [9].

Determination of the percentage of non-filterable cells in erythrocyte suspensions. The percentage of non-filterable cells in a suspension was determined using the above-mentioned filtrometer. Knowledge of t_{b0} allows one to find the mean number of pores in a given filter. According to the

Poiseuille's law, $Q/t_{b0} = N_0 p\pi r^4/8\mu h$, where the passing volume $Q = 250$ μl, the mean pore radius $r = 1.55$ μm, the filter thickness $h = 7$ μm, the hydrostatic pressure $p = 6$ cm H_2O and the water viscosity at 20°C $\mu = 0.01$ g/cm s. This gives $N_0 = 1.7 \cdot 10^6/t_{b0}$ pores.

If a small volume of the suspension under study with the known number of cells n is passed through a filter and then a much greater volume of the buffer is passed through the same filter, a situation is possible when the number of occupied pores and, consequently, the rate of buffer filtration will become constant. By estimating the number of occupied pores and having the total quantity of cells passed through the filter, one can determine the percentage of non-filterable erythrocytes.

To this end, after filtration of a fixed volume of the suspension with known cell concentration, we multiply passed the buffer through the same filter and measured successively the passage time of each subsequent buffer portion t_{b1}, t_{b2}, etc. A constant value of t_{bf} is established after the passage of 3–5 ml of buffer through the filter. The magnitude of t_{bf} is inversely proportional to the number of free pores. Therefore, the percentage of clogged pores R after the passage of n erythrocytes through the filter is determined as $R = (N_0 - N_f)/N_0 = (t_{bf} - t_{b0})/t_{bf}$. Then the next portion of the suspension under study was passed through the same filter and after multiple filtrations of buffer portions a new value of t_{bf} and respectively new R were derived. This procedure was repeated several times to reach gradual clogging of 40–60% of pores in each filter.

It is essential that the filter was never dry during all these filtrations, i.e., a thin layer of liquid was always available above it. Otherwise, the results would be distorted because the cells in pores would be under the action of an additional pressure created by surface tension forces.

RESULTS AND DISCUSSION

Figure 1 shows the dependence of the relative content of clogged pores R on a filter on the number of erythrocytes applied to the filter for suspensions varying in the content of non-filterable cells. Curves *1* and *2* were plotted from the results of passage of erythrocyte suspensions treated with 0.1% glutaraldehyde through the M-3 and Nuclepore filters, respectively. Curves *3* and *4* were obtained after the passage through the M-3 filter of suspensions of intact donor erythrocytes in the isotonic medium containing respectively 17.5 and 7% of glutaraldehyde-treated cells. Curve *5* corresponds to the suspension of intact donor erythrocytes in the hypotonic medium with an osmolality of 235 mOsm/kg.

All the curves shown in Fig. 1 have a characteristic shape caused by the difference in the diameter of pores in the filter and also by overlapping pores. The initial segment of each curve ($0 < R < 0.1-0.2$) is determined by the

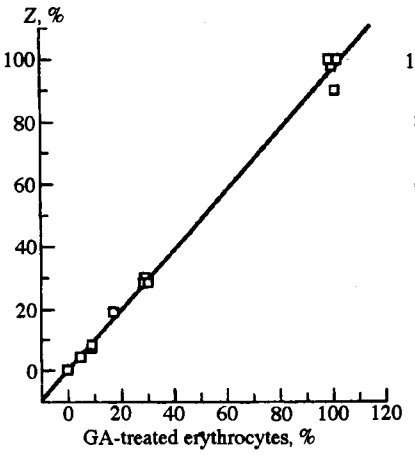

Fig. 3

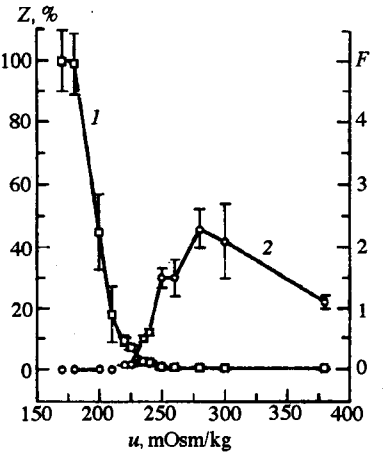

Fig. 4

Figure 3. Percentage of non-filterable erythrocytes in (Z, %) vs. the relative number of glutaraldehyde (GA)-treated erythrocytes added to suspensions of intact cells in the medium with osmolality $u = 310$ mOsm/kg.

Figure 4. Dependence of the percentage of non-filterable cells (*Z*, %) in suspensions of erythrocytes from the blood of healthy donors (*1*) and filtrability index (*F*) of such suspensions (*2*) on the osmolality of resuspension medium (*u*).

plugging of the smallest pores and has the largest slope. (According to our data, 10% of the mean initial number of pores of the M-3 type nuclear filters are the pores preventing even the filtration of normal erythrocytes.)

The major portion of pores with the mean diameter for this type of filters characterizes the linear segment of the curve ($0.2 < R < 0.6$). Then the curve becomes ever more flattened that corresponds to the clogging of pores with the largest diameter and overlapping pores. To determine the efficiency of the clogging of pores with the mean diameter, we used the linear segment of the R vs. n curve ($0.2 < R < 0.6$).

The absolute quantity of clogged pores was obtained as a product of R by N_0 for each filter. Figure 2 shows the linear segments of the curves reflecting the dependence of RN_0 on the number of erythrocytes on a filter. It is seen that at such standardization the curves corresponding to glutaraldehyde-treated suspensions (Fig. 1, curves *1* and *2*) prove to be parallel (Fig. 2, curves *1* and *2*), the slope of these curves k being equal to unity. (Note that the initial quantity of free pores N_0 on the M-3 filter is three times as low as that on the Nuclepore filter.) Thus, as one should expect, the erythrocytes treated with glutaraldehyde in the hypotonic medium totally clog the pores of both types of filters, i.e., the suspension contains 100% of non-filterable cells. Curve *3*

results from the passage through the M-3 filter of normal intact erythrocytes resuspended in the isotonic medium with 29% of glutaraldehyde-treated cells.

Other curves shown in Fig. 2 were plotted from the results of passage of suspensions of normal intact erythrocytes in hypotonic media through the M-3 filters. Each curve is characterized by the slope equal to the percentage of non-filterable cells in a suspension under these conditions ($k = Z$, %/100).

Figure 3 shows a good correlation between the relative content of glutaraldehyde-treated cells in a suspension of normal intact erythrocytes and the percentage of non-filterable cells (Z, %) measured by the above-described method.

The filtrability of erythrocytes is known to decrease with the lower medium osmolality [13]. This phenomenon is related, at least in part, to the increasing percentage of non-filterable cells in the suspension studied. Table 1 presents the results obtained at different osmolalities. They include the percentages of non-filterable cells Z, %) in suspensions of washed erythrocytes from the blood of 8 healthy donors and the filtrability index (F) of these suspensions. These data show that the magnitude of Z varies little being on average 0.4% in a wide range of u from hypertonic to isotonic values. This background level of Z seems to be determined by the admixture of non-washed-out leukocytes, platelet aggregates and strongly modified erythrocytes [9]. Upon further osmolality decrease the magnitude of Z increases more and more sharply reaching its maximal value of 100% near 180 mOsm/kg. Comparison of corresponding Z and F values shows that with the osmolality decrease the 1% suspension of erythrocytes virtually ceases to pass through a filter when the percentage of non-filterable cells in it becomes more than 2–3%. In this case the number of non-filterable cells brought onto the filter in the process of measurement is approximately equal to the number of filter pores N_0. The data of Table 1 are represented graphically in Figs. 4 and 5.

Figure 4 demonstrates the relationship between the dependences on osmolality u of the filtrability index F (Fig. 4, curve 2) and of the percentage

Table 1. Percentage of non-filterable cells (Z, %) and filtrability index (F) at different values of resuspension medium osmolality (u) in suspensions of erythrocytes from the blood of 8 healthy donors.

v	u, mOsm/kg	Z, %	F	v	u, mOsm/kg	Z, %	F
1	170	100	0	6	235	2.5±0.5	0.5±0.06
2	180	100	0	7	250	0.7±0.3	1.5±0.2
3	200	45±12	0	8	260	0.6±0.3	1.5±0.5
4	210	18±9	0	9	300	0.4±0.3	2.1±0.6
5	220	9±1.4	0.06±0.05	10	380	0.4±0.3	1.1±0.05

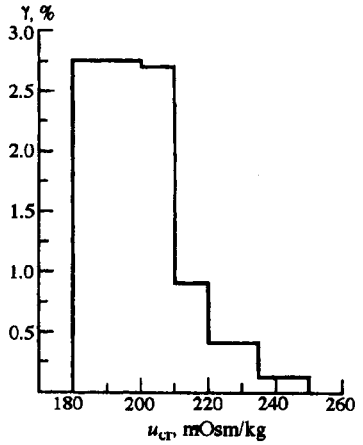

Fig. 5

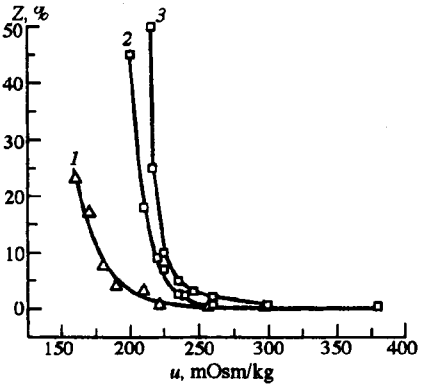

Fig. 6

Figure 5. Distribution of cells in a suspension of erythrocytes from the blood of healthy donors as a function on u_{cr} (for comments see the text).

Figure 6. Influence of medium osmolality (u) on the percentage of non-filterable cells (Z, %) in suspensions of erythrocytes from: *1*, patient with xerocytosis; *2*, healthy donor; *3*, patient on chronic hemodialysis because of acute renal insufficiency.

of non-filterable cells Z determined by the method we propose (curve *1*). Curve *1* presenting the dependence of Z on u is by definition the cumulative distribution of erythrocytes in u_{cr}. A representative distribution of erythrocytes from a normal donor in u_{cr} is shown in Fig. 5 where $\gamma(u_v < u < u_{v+1}) = [Z(u_v) - Z(u_{v+1})/(u_{v+1} - u_v)$. The maximum of the distribution is at u_{cr} = 190–200 mOsm/kg. Having u_{cr} for a filter with known parameters and isotonic volume of erythrocyte V_{iso}, one can easily determine the area of cell surface S and respectively the ratio S/V_{iso} [11]. The results cited above imply that most of normal erythrocytes (mean volume 90 μm^3 [14]) have S/V_{iso} = 1.72–1.68 μm^{-1} which correlates well with the literature data [15]. The half-width of the distribution of erythrocytes in u_{cr} (Fig. 5) is 20–30 mOsm/kg for different samples of donor blood. Apparently, the distribution of erythrocytes in u_{cr} reflects their distribution in the S/V_{iso} ratio and is analogous to the distribution over the "lysis tonicity" reported in [16]. However, it is still unknown how the distribution of suspended erythrocytes in u_{cr} corresponds to their distribution in volume and other parameters. The character of the distribution of erythrocytes in u_{cr} varies in certain pathologies and damaging impacts. Figure 6 shows segments of the cumulative distributions of erythrocytes in the blood of a patient with xerocytosis (curve *1*), in the normal donor blood (curve *2*) and in the blood of a patient with renal

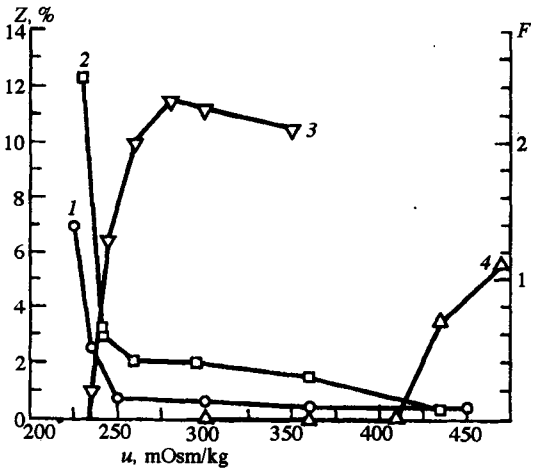

Figure 7. Influence of medium osmolality (u) on the percentage of nonfilterable cells (Z, %) (*1*, *2*) and filtrability index (F) (*3*, *4*) for suspensions of erythrocytes from the blood of normal donors (*1*, *3*) and ATP-depleted erythrocytes (*2*, *4*).

insufficiency maintained on hemodialysis (curve *3*). The shift of curve *1* to the left and the shift of curve *3* to the right compared to the normal distribution are indicative of a considerable increase in the S/V_{iso} ratio of erythrocytes under xerocytosis [17] and a slight decrease of this ratio in the patient with renal insufficiency.

Figure 7 shows the results of two assays with ATP-depleted erythrocytes. Washed erythrocytes were incubated in glucose-free HEPES buffer for 20 h at 37°C. After triple washing of these erythrocytes in the same buffer, the dependence of Z and F on the resuspension medium osmolality was studied. As seen (Fig. 7), the ATP-depleted erythrocytes are not filtered up to $u = 410$ mOsm/kg. A further increase of u leads to a gradual growth of F (curve *4*). The effect of filtrability decrease of ATP-depleted erythrocytes is well known [18, 19]. However, the authors who studied such erythrocytes using a CTTA analyzer failed to reveal their difference from the normal cells with regard to the filtration rate of individual cells through the pores 5 μm in diameter [20]. Comparison of the dependences of Z on u (cumulative distributions in u_{cr}) indicates that the larger part of erythrocytes in the ATP-depleted suspension do not differ strongly in S/V_{iso} from that of normal cells. In this case the significant shift of the filtration curve to the right (curve *4*) is explained by the presence in the ATP-depleted erythrocyte suspension of a small (about 2%) fraction of strongly modified cells for which $u_{cr} > 410$ mOsm/kg.

Thus, by applying the simple method described above and using an ordinary filtrometer, one can derive new information on the distributions of

rheological properties of erythrocytes in suspensions. This method makes it possible to reveal in suspensions minor fractions of erythrocytes with modified properties accounting for fractions of percent of the total cell number. Such an information can be helpful in clinical studies and also be used for the interpretation of kinetic filtration curves.

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