Discrimination between orientation and elongation of RBC in laminar flow by means of laser diffraction

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ABSTRACT

The elongation curve of RBC as determined by rheoscopy or ektacytometry (laser diffraction) resembles a rectangular hyperbola. The experimental data obtained so far included too large errors of measurement to allow precise mathematical description. The combination of laser diffraction with image analysis has improved ektacytometry considerably, such that the error of measurement is reduced to less than 0.5%. In laminar flow RBC of healthy donors are elongated elliptically ($p \leq 0.001$). Using the precise data of elliptical deformation, the elongation curve can be described to be hyperbolic. Hence, the double reciprocal plot gives a linear curve which - over a wide range of shear stress (15 to 500 dyn/cm^2) - fits well the experimental data ($r \geq 0.99$: $p \leq 0.001$). The stress strain characteristics (i.e. elongation curve) can be described by two parameters: maximum elongation (E_{max}) and the shear stress needed for half-maximum elongation (K_E). Mechanical stress only slightly reduces E_{max} but significantly shifts K_E to higher values. Hyper-osmolarity decreases E_{max} and increases K_E. Mild hypo-osmolarity (>225 mOsm.) increases E_{max} and decreases K_E, whereas strong hypo-osmolarity (≤225 mOsm.) decreases E_{max} and further decreases K_E

Inclusion of elongation data measured at shear stress < 15 dyn/cm^2 detoriates the linear correlation. The elongation coefficients measured for low shear stress are higher than calculated by linear regression. The same holds true for range of shear stress between 15 and 25 dyn/cm^2 if applied to RBC in hypertonic solutions or to cells which underwent mechanical stress. Further analysis of the laser diffraction patterns led us to suggest that at low shear stress the RBC become more or less oriented thereby presenting their side aspect (2.2 µm ·8 µm) to the laser beam. The diffraction pattern then resemble to those of elongated RBC. At shear stress exceeding 15 dyn/·cm^2 the RBC rotate by 90° presenting their circular aspects (∅ 8 µm) which then become elliptically deformed by shear .

2. INTRODUCTION

Three closely related experimental set-ups have been established to quantitatively estimate deformability of human RBC: rheoscopy, ektacytometry and laserdiffractoscopy. RBC are exposed within a viscometer to defined shear stress, which may be changed by varying the viscosity of the suspending fluid or the shear rate applied. Rheoscopy includes photomicrographic imaging in order to evaluate the geometric parameters of single cells. In ektacytometry the cell geometry is calculated via analysis of intensity at distinct points within the laser diffraction pattern. Laserdiffractoscopy uses the entire information content of laser diffraction patterns by means of image analysis and therefore is more precise than ektacytometry and less time consuming than rheoscopy. The flexibility of RBC determined by these methods is usually attributed to a single parameter E (or DI), derived from the quotient of the major and minor axis of elliptically deformed RBC (see figure 2). The stress strain characteristic (elongation curve) of whole RBC can be described plotting the elongation coefficient E versus shear stress.

In the present paper an attempt is made to formulate the stress strain characteristics mathematically in order to reduce its information content and to separate elongation of RBC from the orientation of the cells in the laminar flow.

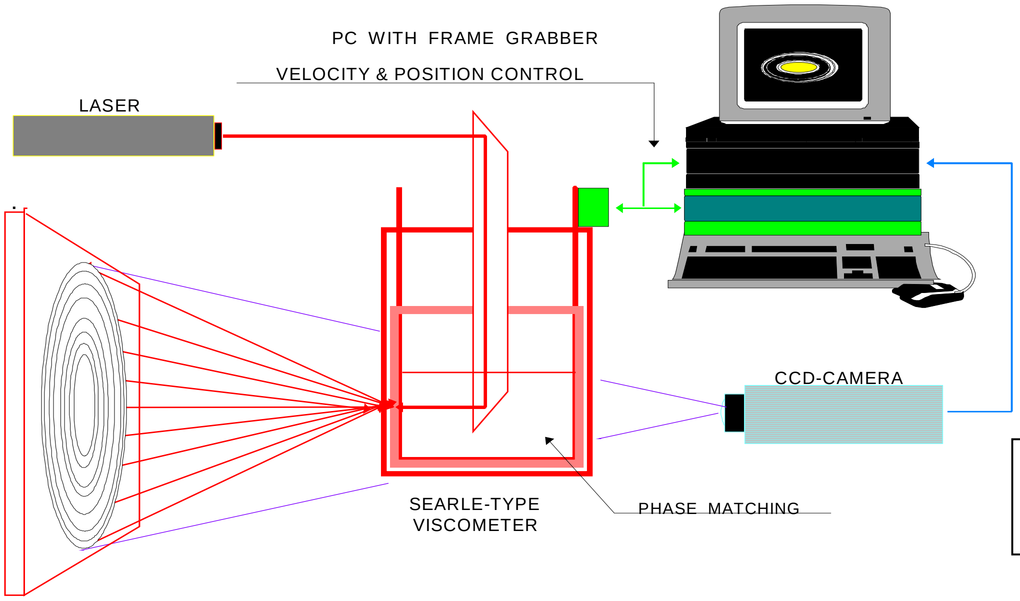


Fig. 1: Experimental set-up of the laser diffractoscope

3. MATERIAL AND METHODS

3.1. Blood preparation and solutions

Blood was drawn from the cubital vein of healthy volunteers in heparinized tubes (Vacutainer, Becton & Dickinson). The suspending medium contained 210 g/l dextran (MW 60000, Schiwa) in a MOPS buffered saline (140 mM/l NaCl, 5 mM/l KCl, 5mM/l glucose, 3 mM/l MOPS buffer). The pH was adjusted to 7.4, the viscosity of the suspending medium (22 ± 1 cpoise) was controlled with a Hoeppler viscometer (Haake), the osmolarity (300 ± 5 mOsm) by micro osmometry (Knauer). For measurement of RBC deformability 4 ml of the dextran containing solution and 0.2 ml of blood were gently mixed and filled in the laserdiffractoscope. All measurements were carried out at room temperature.

3.2. The apparatus

The method to measure elongation of RBC by means of laser diffraction has already been described previously [2, 3, 5, 6, 11, 14]. Figure 1 shows schematically the experimental set-up. A viscometer is used to produce well defined shear stress within a gap of 0.5 mm between two transparent cylinders. The rotating inner cylinder (R_i = 24.5 mm) offers the advantage of effortless gap filling and emptying and, most importantly, the absence of lens effects of the outer (R_o = 25 mm), non-rotating cylinder (Searle-system) due to its plane front face. The inner cylinder can be driven with velocities in the range of 0 to 500 rpm, corresponding to shear rates between 0 and 2620 /s. The velocity and the position of the inner cylinder is controlled by a photo detector, in order to gain the current shear rate allowing to shoot the diffraction pattern at a defined position. The laser beam (He-Ne-Laser, 20 mW) is passed into the rotating cylinder via an aluminized dove prism. Undisturbed transmission from the prism to the gap is achieved by phase-matching. The gap is filled with RBC, suspended in an isotonic solution of high viscosity. In this manner, the RBC are exposed to variable shear stress, depending on solution viscosity and shear rate. The diffracted laser beam is projected on a reflection screen and photographed with a CCD camera. The BAS video signal is digitized ("Fast screen machine II", 8 bit, real-time, maximum resolution 736 x 560 pixels) and transferred to a 486 PC for display and further analysis.

As in ektacytometry [5, 6, 11] the image analysis is based on light- intensity measurement. After A/D conversion discrete intensity values are attached to each pixel, which over a range of 256 intensity steps are linearly related to the intensity of incoming light. The main issue of laser diffractoscopy is to extract intensity information from up to 412.160 points. This offers the opportunity to compare light intensity at different loci and to evaluate areas of selected intensity forming circles or ellipsoids of equal light intensity. These isointensity lines represent the geometric form of RBC. Elongated RBC diffract collimated light according to their shape. Circular RBC yield a circular diffraction pattern, elliptical RBC yield an elliptical pattern with the same eccentricity, but rotated by 90°.The diffraction intensity distribution becomes wider as particles get smaller, the minor axis of an ellipsoid isointensity line corresponding to the major axis of the sheared RBC and vice versa. Since the distribution of points of equal intensity form circles or ellipses a linear correlation can be applied (using the square of loci of each pixel) to determine the parameters of ellipses.

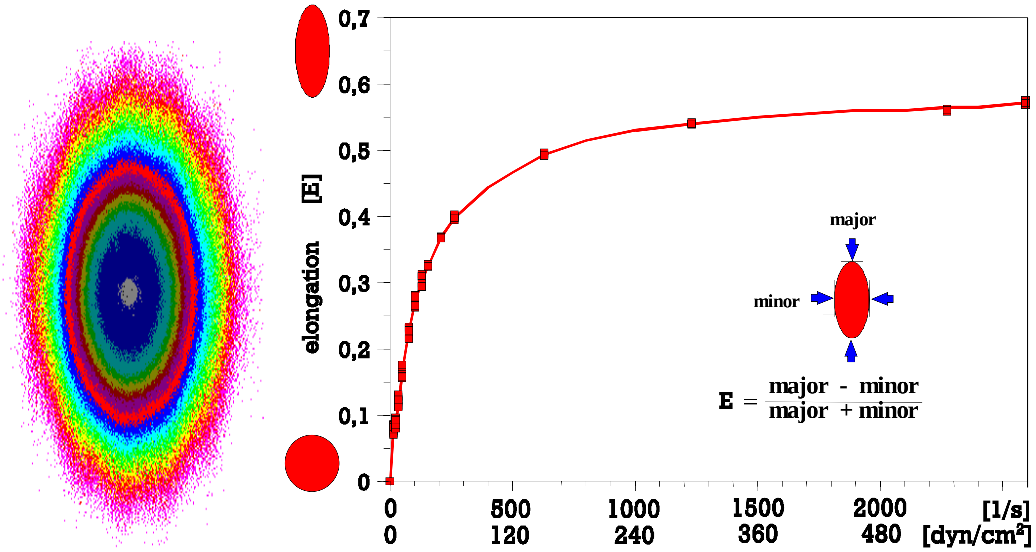


Fig. 2: Example of a diffraction pattern of elongated RBC (left). The elongation curve (right) is obtained by plotting the elongation coefficient "E" versus shear rate [1/s] or the force produced within the gap of the viscometer (shear stress [dyn/cm^2]).

From each diffraction pattern (figure 2) a series of isointensity lines and the corresponding E values are calculated. Due to the noise of CCD chips, the elongation coefficient [E] calculated for the low intensity range includes rather high errors (correlation coefficients for isointensity lines < 0.9000). Close to the center of the diffraction image (high intensity range) the light of the non-diffracted laser beam adds a circular intensity distribution to the elliptical one. Consequently, the inner and outer isointensity lines are not included to determine the average E (with standard deviation SD) for each diffraction picture. The method error for this determination of RBC elongation has been previously shown to be about 1% (variation coefficient)[3].

In laminar Searle- (or Couette-) flow the shear stress applied is a function of the viscosity of the suspending fluid, the rotation speed of the outer cylinder and the geometry of the cylinders. From the analysis of error of measurement it emerged that at low shear rates, which correspond to the steep part of the elongation curve (see figure 2), small changes of the gap width or unsteady cylinder rotation are the main cause of errors[2]. Therefore a computer assisted control of motor speed and , most importantly, a position control device were introduced to avoid small fluctuations of gap width and to monitor the rotation of the cylinder continuously. Such, the variation coefficient could be reduced to less than 0.5%.

4. RESULTS AND DISCUSSION

Some attempts have been done to linearize or to describe the stress strain characteristics of whole RBC mathematically. However, the data used so far had rather large error of measurement; in rheoscopy mainly due to the limited optical (spatial) resolution, in ektacytometry due to erroneous calculation of elongation at high shear stress and problems related to working slightly off axis. Hence, using a more precise set-up allowing to get most confidential data a new attempt of formal description of the stress strain characteristic of whole RBC has been undertaken.

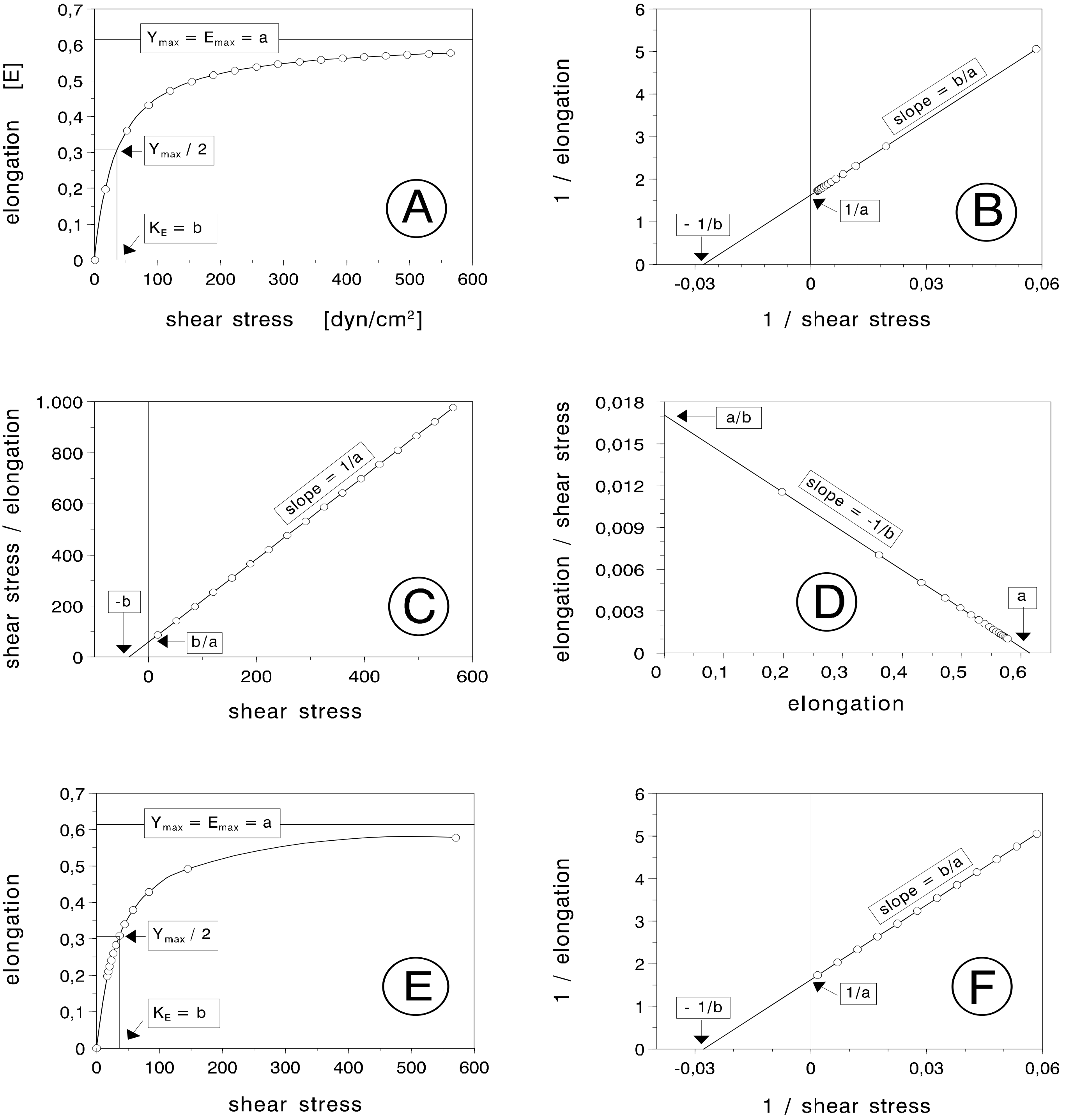


Fig. 3 : Typical elongation curve of human RBC using even spacing of shear stress (A) or even spacing of 1/shear stress (E). Examples of linearization : double reciprocal (B, F), Y-reciprocal (C) and X-reciprocal (D) plot.

The so-called elongation curve, measured by means of rheoscopy or analysis of laser diffraction patterns (ektacytometry, laserdiffractoscopy) resembles a rectangular hyperbola. The semilogarithmic plot of Y against log X often used [7, 8, 12] gives a nonlinear function. It does not allow to estimate Y_{max} accurately but it is possible to find the value of X corresponding to half maximum Y, for this marks the inclination point in the curve. The advantage of the semilog plot is only its capability of presenting data covering several orders of magnitude of X. On the other hand, there are three non logarithmic linear plotting forms of rectangular hyperbola [10] (see figure 3) :

- the double reciprocal most commonly used in enzyme kinetic studies and is called the Lineweaver-Burk plot)
- the Y-reciprocal
- the X-reciprocal

These three forms of linearization are not equivalent and each have advantages and limitations.

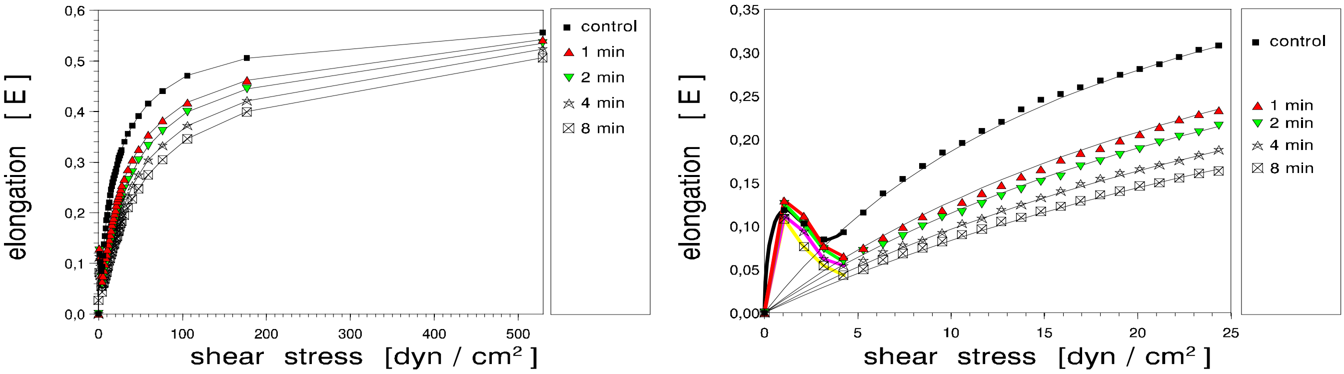
1. When the values of X, the independent variable are equally spaced the linearization can change the relationship. The double-reciprocal (figure 3B) and the X-reciprocal (figure 3D) plots are notorious for that. Points of low X become widely spaced, whereas those at high X being closely bunched. In this situation the calculation of linear correlation and the placement of the line is extremely sensitive to those Y-values having the smallest X. In consequence one has to introduce statistical weighting procedures or - if experimentally possible - to choose X such that the points on the 1/X-axis are rather evenly spaced.
2. Mixing X and Y (X- and Y-reciprocal plots) introduces further statistical problems, since the uncertainty of the dependent variable Y is greater than that of the independent variable X.
3. In the Y-reciprocal plot (figure 3 C) the spacing of X values is retained. The X-reciprocal plot provides a closed scale representation of Y (the to other forms are open ended) but has the disadvantage of having independent variable on both axis.
4. The double reciprocal plot has the advantage of leaving the variables X and Y separated on abscissa and ordinate. From point of view of statistics the double reciprocal plot is superior if experimental conditions can be chosen providing an even spacing of 1/X.

Figure 3 E,F shows the data of an experiment in which the shear stress applied was chosen to fulfil the requirements of even spacing in a double reciprocal plot The reciprocal value of the intercept of the linear correlation with the Y-axis is equal to the maximum elongation ($1/a = E_{max}$) and the intercept with the X-axis gives the shear stress needed for half maximum elongation ($1/b = K_E$). Regression analysis of experimental data obtained with blood of healthy donors yielded highly significant correlations in every case ($r > 0.99$; $p \leq 0.001$)

However, in order to get such good correlations one has to discard those data corresponding to shear rates < 15 dyn/cm^2. Figure 4 (top, right) gives a closer look on course of the elongation curve in the low shear stress range. Even under best control conditions (fresh blood of healthy donors, pH 7.4, isotonic solution, avoidance of any other stressing factor) there exists an - inter-individually more or less expressed - initial hump within the curve. This hump, which has already been seen in elongation curves determined in low viscosity media[13], is suspected to be caused by cell orientation. It is also hidden in the curves determined in high viscosity media, obviously distorting the hyperbolic function, which in turn is mainly related to red cell elongation.

As shown recently RBC exposed to shear stress exceeding a threshold of 200 dyn/cm^2, become rigid as depending on duration and extent of the mechanical stress [1, 4, 15]. Analysis of the stress strain characteristics (figure 4) confirmed our previous observation that maximum elongation is not considerably affected. The main effect of mechanical stress (8 minutes 540 dyn/cm$^{2)}$ is the increase of K_E from 20.2 to 53.5 dyn/cm^2. The position and the amplitude of the hump seems not to be different if compared with that under control conditions.

Supposing that a) the initial hump is somehow related to RBC orientation in laminar flow and b) cell orientation is a function of their shape, changes of osmolarity are expected to produce drastic effects via shrinking (loss of water) or sphering (uptake of water). Figure 5 demonstrates the effects of hyperosmolarity (adding NaCl; keeping KCl, glucose, buffer, pH and dextran constant). In contrast to mechanical stress hyperosmolarity strongly reduces E_{max} and increases $K_{E.}$ At osmolarities of the surrounding fluid > 398 mOsm the linear regression did not fit the data sufficiently, such that E_{max} and K_E could not be determined.



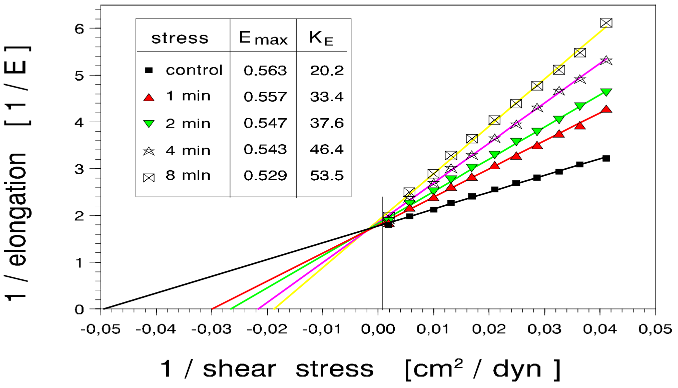
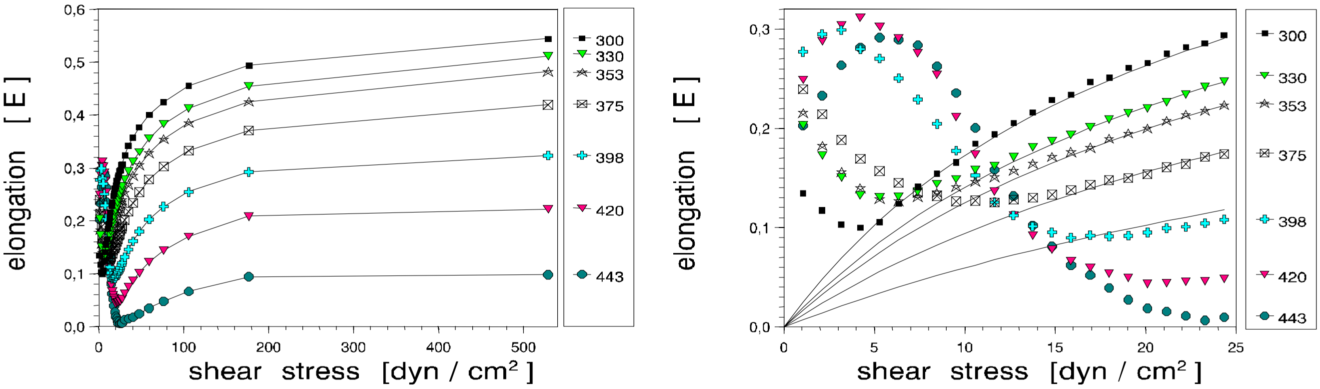


Fig. 4: Effect of mechanical stress (540 dyn/cm², duration 1, 2, 4 and 8 min) on the elongation curves of human RBC (top left). Linearization using double reciprocal plots of data corresponding to shear stress < 25 cyn/cm² (top right). Note that, except a small initial hump, the functions obtained by linear correlations (r >0.998, bootom left) also fit the experimental data corresponding to low shear stress quite



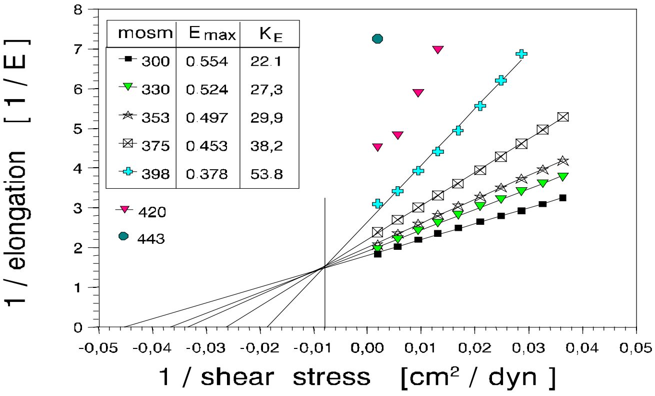
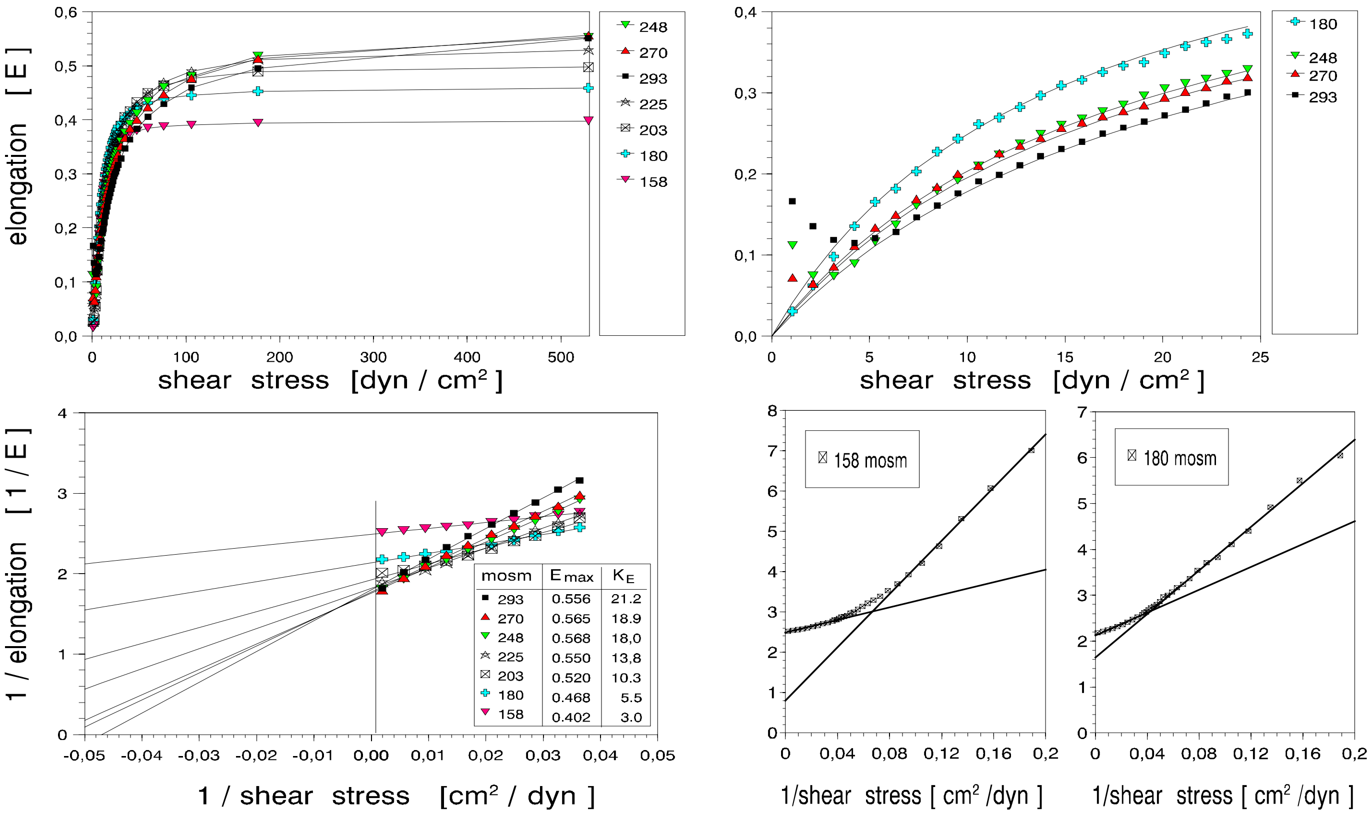


Fig. 5: Effect of hyperosmolar stress (330 - 443 mosm.) on the elongation curves of human RBC (top left). Linearization using double reciprocal plots of data corresponding to shear stress >25 dyn/cm² (bottom left). Elongation curves for shear stress < 25 dyn/cm² (top right). Note the increase of the initial hump with increasing osmolarity. The functions obtained by linear correlation (r >0.998; bottom left) fit the data corresponding to low shear stress rather poorly. At osmolarities >385 mosm. they do not fit the experimental data at all

The right part of figure 5 demonstrates the action of hyperosmolarity on the course of the initial hump. With increasing osmolarity its amplitude increases up to an "elongation coefficient [E]" of > 0.3. Comparing the function (calculated by linear regression; figure 5 bottom, left) with the experimental data corresponding to low shear stress it becomes obvious that with increasing osmolarity congruity is reached at higher shear stress. In the case of 420 and 443 mOsm concordance was not at all obtained. Hence, under extreme experimental conditions, linear regression of double reciprocal data sets may give erroneous results, since the mechanisms underlying the initial "local" hump stretch to a rather large part of the elongation curve.

Hypo-osmolarity (reducing NaCl; keeping KCl, glucose, buffer, pH and dextran constant) in some respects induces opposite effects (see figure 6). Mild hypotonic (≥ 248 mOsm) solutions increase E_{max} whereas at lower osmolarities E_{max} decreases. But, in every case hypo-osmolar conditions reduce $K_{E.}$.At the top (right) of figure 6 the low shear stress range of the elongation curve determined under hypotonic conditions is shown. It clearly demonstrates that the initial hump disappears with decreasing osmolarity. Down to an osmolarity of 248 mOsm the functions calculated by linear regression fitted also the data corresponding to low shear stress rather well. In the range between 158 and 225 mOsm the double reciprocal plot of all data (except that of the tiny initial hump) revealed that the stress strain characteristics of RBC under strong hypotonic conditions my described assuming two hyperbolic functions (bottom right of figure 6)



mosm	E_{max}	K_E
■ 293	0.556	21.2
▲ 270	0.565	18.9
▼ 248	0.568	18,0
☆ 225	0.550	13,8
☒ 203	0.520	10.3
✚ 180	0.468	5.5
▼ 158	0.402	3.0

Fig. 6 : Effect of hypotonic stress (248 - 158 mOsm) on the elongation curves of human RBC (top left) Linearization using double reciprocal plots of data corresponding to shear stress > 25 dyn/cm² (bottom left). Elongation curves for shear stress < 25 dyn/cm². Note the loss of the initial hump with decreasing osmolarity. The functions obtained by linear correlation fit well the experimental data (for sake of clearness some curves are not shown). The curve fitting the data of 180 mOsm is derived from regression analysis assuming two hyperbolic functions (bottom right).

Our prime target was not to investigate the influences of osmolarity or mechanical stress on RBC flexibility. The effects of osmolarity[9] and mechanical stress in laminar flow[1, 4, 15] have been extensively described. In principal, our results confirm these data. However, in contrast to Clark et al.[9], the result presented here indicate that mild hypotonic solutions improve the flexibility of RBC.

The main task was to describe the stress strain characteristics of RBC elongated in a viscometer. From the results presented, it is obvious that the elongation curve can be described by a rectangular hyperbola. Furthermore, the double reciprocal plot and regression analysis seem to be useful tools to analyse RBC flexibility, allowing to characterize the viscoelastic properties of whole cells by two parameters E_{max} and $K_{E.}$ Nevertheless it has to be pointed out that under some conditions as severe hypo- or hyper-osmolarity linearization does not fit the experimental data. One can expect that in some hereditary diseases with strange shapes of RBC deviations from the hyperbola will be obtained too.

We suppose that the main cause for the deviation of the curve from a proper hyperbola is related to problems of orientation of the cells in the laminar flow. Figure 7 illustrates our hypothesis.

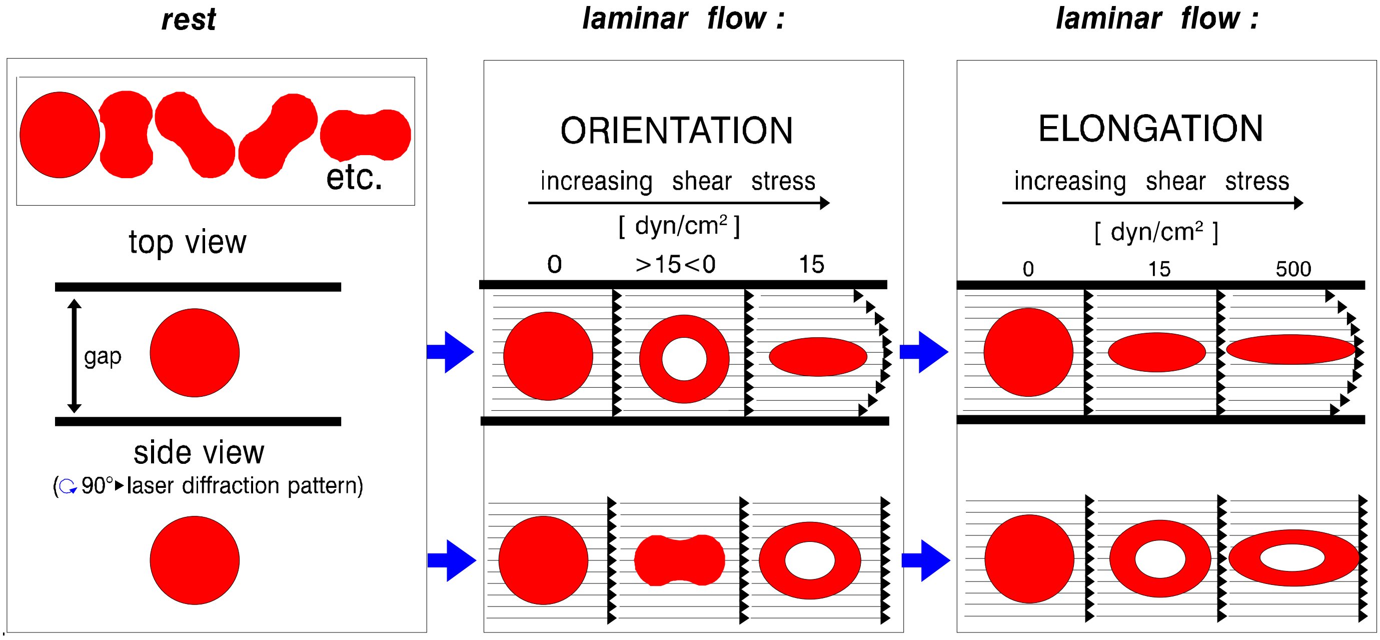


Fig. 7: Orientation and elongation as dependent on shear stress

At rest the RBC present randomly all aspects to the laser beam, which at least will give an average aspect of a round body. At low shear stress some of the cells present their flat side aspect to laser beam deceiving an elongation. The amplitude of the hump, which is based on this, depends on the number of cells orienting in such a way. Under the condition of hypertonic media this type of orientation of exsiccated RBC is rather stable. More shear stress is needed to turn the cells in such a way that they present the laser beam their round side, which then becomes elliptically deformed.

5. ACKNOWLEDGMENTS

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