

Laser diffraction of RBC: the method and its pitfalls

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1. ABSTRACT

The method to determine red blood cell (RBC) deformability by laser diffraction is presented. It combines ektacytometry (laser diffraction) with image analysis. Laser light is passed through erythrocyte suspensions, which are sheared in a Searle like viscometer. The laser diffraction patterns are photographed by a CCD camera and further analysed by a computer. Flexibility is characterized by the quotient of minor and major axes of iso-intensity lines of the elliptically transformed diffraction patterns. The variation coefficient of the measurement is less than 0.5%.

However, one has to face a series of methodical problems : Occurrence of turbulences and heat of shear, control of viscosity and osmolarity of the suspending medium, control of gap width within the viscometer, differentiation between cell orientation and elongation.

2. INTRODUCTION

In 1813 diffraction of monochromatic light was first used to determine RBC diameter¹⁰. In 1975 laser diffraction as a tool to measure RBC flexibility was introduced by the group of Bessis and Mohandas^{3, 4, 6} (so-called ektacytometry). Here RBC, suspended in a high viscosity fluid, are exposed to shear stress, which may be changed by varying the shear rate applied. Flexibility is evaluated by analysis of the intensities at distinct points within the laser diffraction pattern. Reducing the relevant information of a diffraction image to two (or four) points the accuracy of the method is limited.

The present paper describes a system to measure RBC deformability using the conventional ektacytometry in combination with image analysis (laser diffractoscopy^{1, 2, 8, 9}). Though the accuracy of the method has been improved considerably (variation coefficient of the measurement < 0.5%), it still includes some problems.

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 and 3 mM/l MOPS buffer). The pH was adjusted to 7.4, the viscosity of the suspending medium (21-± 1 cpoise) was controlled with a Hoeppler viscometer (Haake), the osmolarity (305 ±5 mOsm) by micro osmometry (Knauer).

Addition of high molecular dextran is expected to do not alter the osmolarity of the solution. From measurements of the osmolarity it emerged, that dextran 60.000 (210g/l) increases the osmolarity by about 10 mOsm. Analysis revealed, that this is due to its content of low molecular sugars and some potassium. Therefore, for each batch of dextran the buffered saline has to be corrected by reducing potassium and sodium.

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 laser diffractoscope. All measurements were carried out at room temperature.

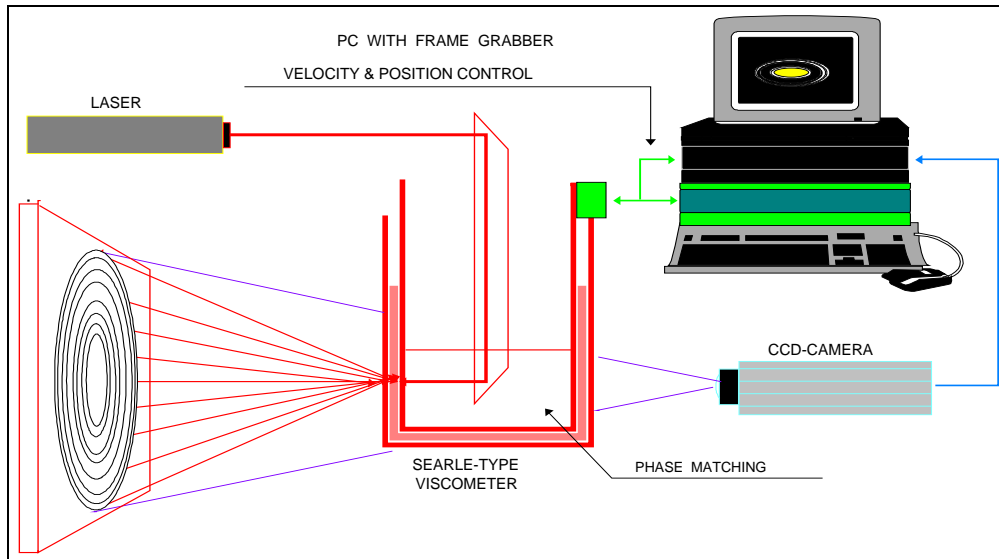


Fig. 1: Experimental set-up of the laser diffractoscope .

3.2. The apparatus

The method to measure elongation of RBC by means of laser diffraction has been described previously^{1, 2, 3, 4, 6, 8, 9}. 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 speed 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.

4. RESULTS AND DISCUSSION

As in ektacytometry^{3, 4, 6} 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 (γ -correction of the CCD camera in switched off position) 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 iso-intensity lines represent the geometric form of RBC. 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.

From each diffraction pattern a series of iso-intensity lines and the corresponding E values are calculated (figure 2 and 3). 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

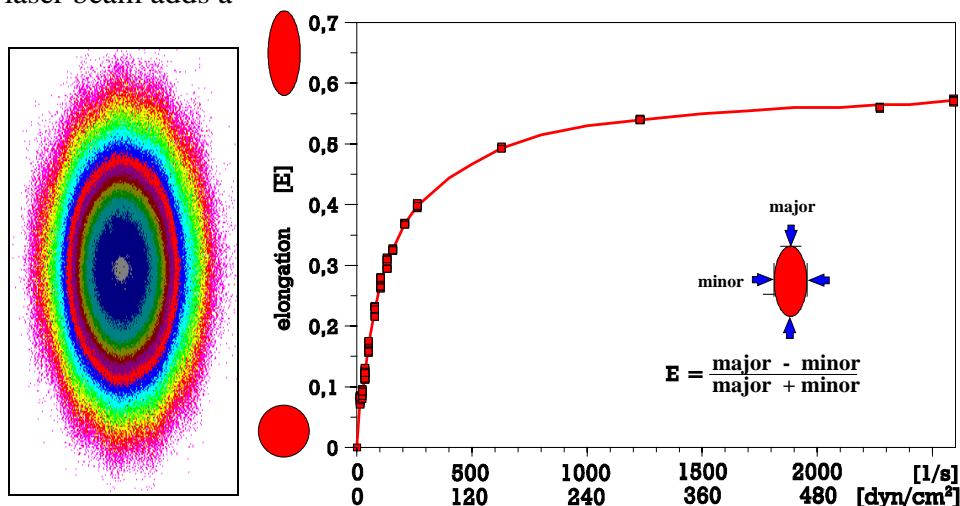


Fig. 2: Example of a diffraction pattern of elongated RBC (left). The elongation curve (right) is obtained by plotting the elongation coefficient [E] versus shear rate [1/s] or the force produced within the gap of the viscometer (shear stress [dyn/cm²]).

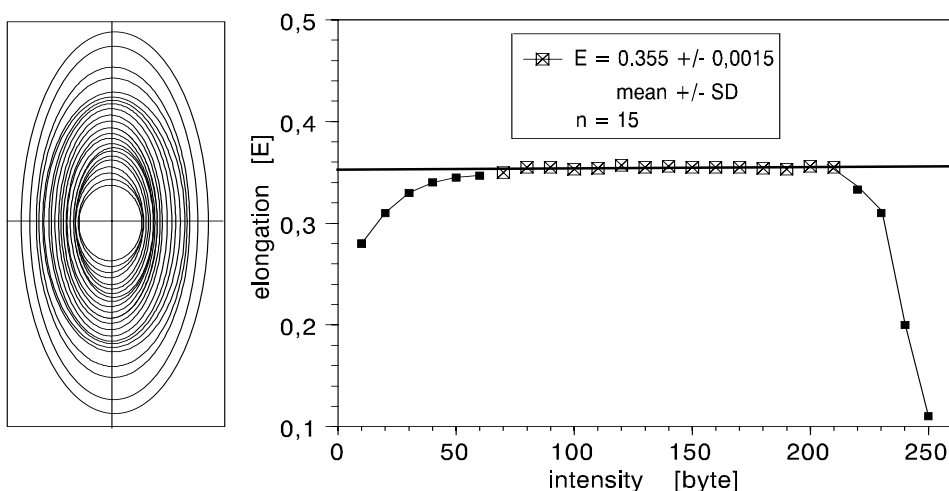


Fig. 3: Series of isointensity lines, calculated from one diffraction pattern (left). Elongation coefficient as depending on intensity selected (right). Only the [E] values represented by the open symbols are included to determine mean elongation [E].

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 shown to be about 1% (variation coefficient)^{1, 2, 8, 9}.

In order to deform RBC shear stress has to be applied, which - in laminar Searle- (or Couette-) flow - is a function of the viscosity of the suspending fluid, the rotation speed of the outer cylinder and the geometry of the cylinders.. Therefore, in order to get accurate results, viscosity, motor speed and gap width have to be kept constant as possible.

The viscosity of the suspending medium is affected by :

- the proportion between the volume of the dextran containing solution and the blood volume added
- the volume of dextran-free saline present after rinsing the gap of the viscometer
- temperature

To determine RBC elongation, usually 200 μ l of blood are gently mixed with 4 ml of high viscosity (dextran) saline and filled in the viscometer As shown in figure 4A, small changes of the relation blood/dextran saline do not considerably alter the viscosity of the suspension. However, rinsing the viscometer with water or isotonic solutions of low viscosity reduces the viscosity of the blood/dextran

suspension severely (figure 5B). Therefore, after cleaning the system with water or saline, one has to rinse the viscometer once or twice with the high the solution of high viscosity.

Performing experiments at room temperature, changes of $\pm 1^\circ\text{C}$ may occur. As figure 5C demonstrates this has only a minor effect on the viscosity.

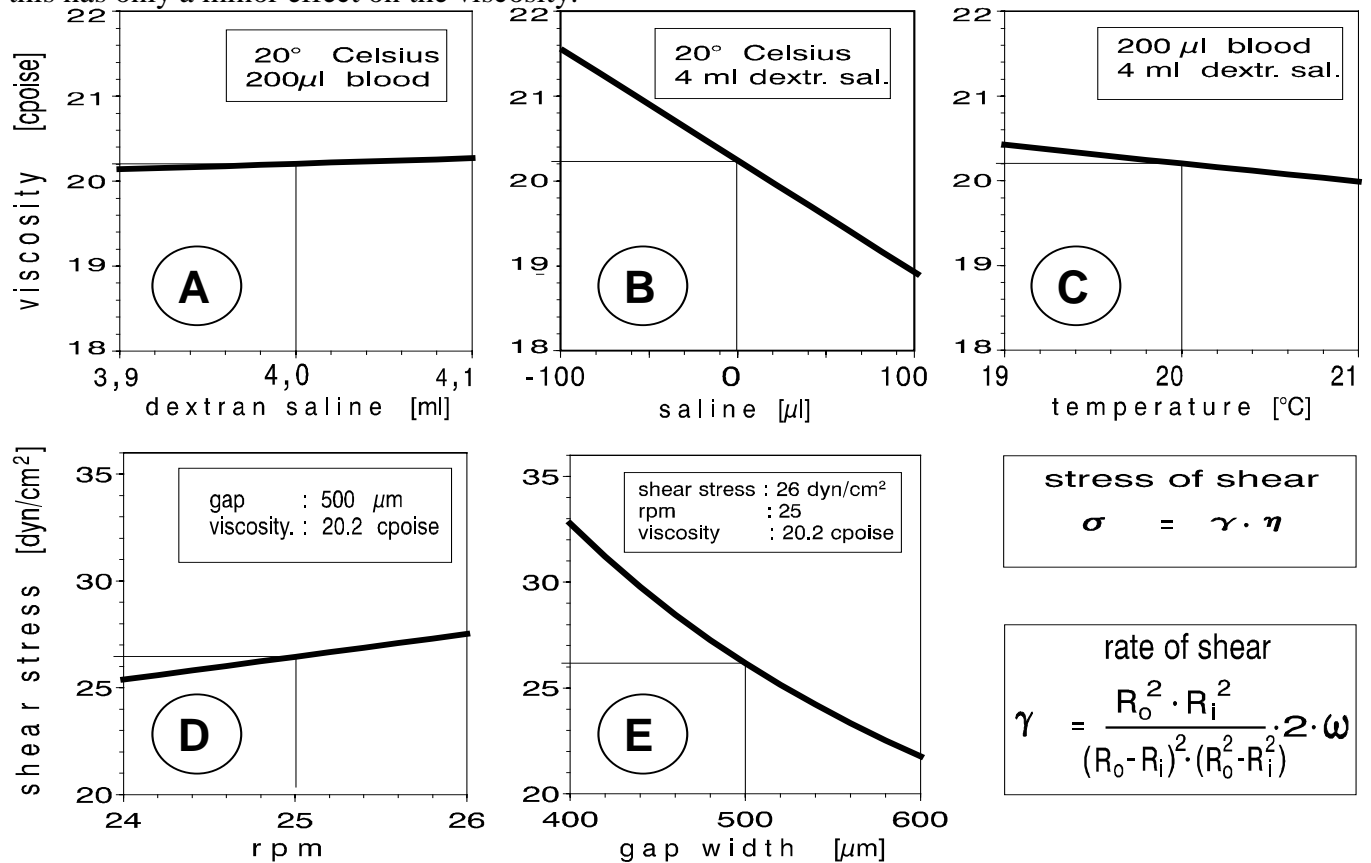


Fig. 4: Effects of changes in : (A) the relation between blood volume to dextran saline, (B) the content of saline of low viscosity after rinsing the viscometer and temperature (C) on the viscosity of the suspension. Calculated changes of shear stress due to fluctuations of motor speed (± 1 rpm, (D)) and gap width ($\pm 100 \mu\text{m}$, (E)). On order to get precise results, rinsing with "low viscosity saline" should be avoided and the gap width controlled carefully. For explanation of variables and constants see figure 6.

The shear stress applied is the product of the viscosity of the suspending fluid and shear rate. The latter is a function of the rotation speed of the outer cylinder and the geometry of the cylinders (for equations see figure 4). From the analysis of error of measurement it emerged two factors may introduce errors :

- small changes of the gap width and
- unsteady cylinder rotation

Within the system described here, a dynamo controlled DC-motor is used to drive the inner cylinder. From rpm measurements it emerged that the rotation speed varies about ± 1 rpm. As shown in figure 4D this affects the shear rate calculated rather little if the rotation speed is set to $^3 25$ rpm. Since the relative error considerably increases lowering the rpm, one has to control the motor speed accurately, whenever measuring RBC elongation at very low shear stress. Our attempts to use a stepping motor were disappointing (vibrations, interferences). Therefore we decided to monitor the rpm by continuous measurement of the (AD-converted) voltage of a dynamo connected to the DC motor.

The main source of false calculation of shear stress is based on small fluctuations within the gap between the two cylinders. These are due to not perfectly centered cylinders, insufficient surface finishing or dirty surfaces (thin layers of fibrin or dry dextran etc.). As the calculation in figure 5E

demonstrates small changes of gap width ($\pm 100 \mu\text{m}$) considerably alter the shear rate. Therefore not only a computer assisted control of motor speed, but also a position control device was introduced, allowing to grab images at selected positions of the cylinder. Such, circumventing the small fluctuations of gap width and monitoring the rotation of the cylinder continuously, the variation coefficient of the measurement of RBC elongation could be reduced to less than 0.5%.

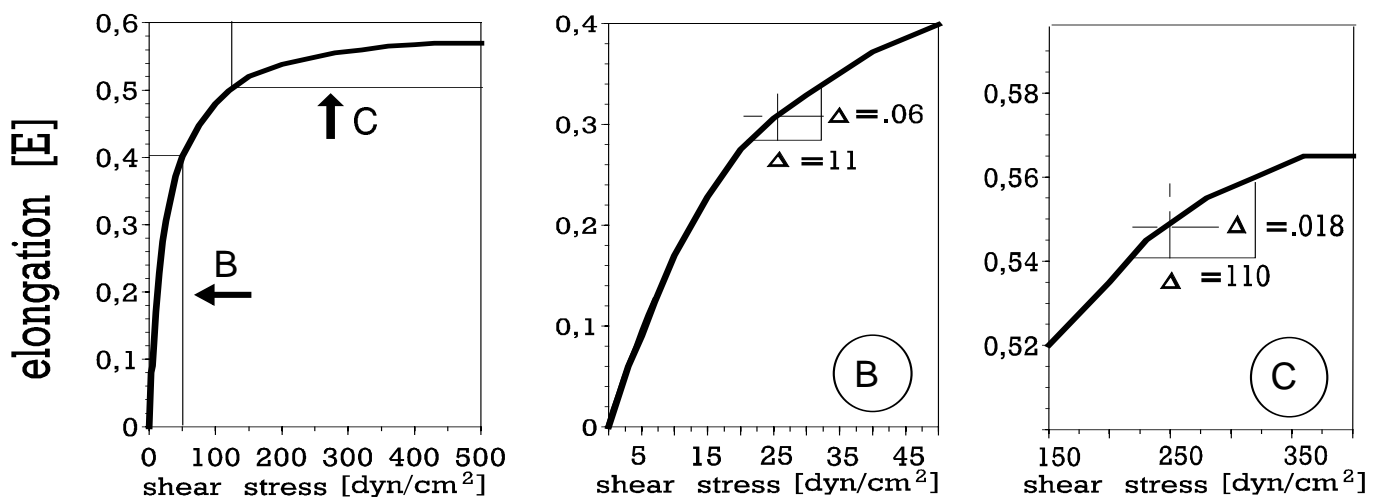


Fig. 5 : Effect of a change of gap width ($\pm 100 \mu\text{m}$) on the elongation coefficient [E] at a mean shear stress of 26 dyn/cm² (B) and 260 dyn/cm² (C).

The elongation curve (figure 2) is a non linear function. Consequently, the error of measurement due to fluctuations of shear stress at a given shear rate may be either negligible or momentous. In the range of low shear stress (e.g. at 26 dyn/cm², see figure 5B), where the curve is rather steep the difference in elongation [E] measured may be rather high (about $\pm 20\%$). In its flat part (e.g. at 260 dyn/cm², see figure 5C) the same fluctuation of gap width results in a trifling change of E (about $\pm 3\%$). In summary : motor speed and gap width have to be carefully controlled, whenever elongation of RBC is determined at a low shear rate.

In viscometry it is well known that viscosity may be underestimated due to the development of heat, which in turn is mainly determined by the square of shear stress. Figure 6 shows the calculations for the system presented here, assuming either no heat loss by dissipation or perfect heat loss via the outer cylinder. Under worst conditions (shear rate 2600/s; no dissipation) an increase of temperature of about 1°C can be expected. Control measurements proved that in the set-up used the shear heat is partially dissipated via the material of the cylinders (Plexiglas \O).

As shown above (figure 4C) an increase in temperature of 1°C reduces viscosity only by 1 cpoise. In addition, one has to keep in mind that concomitantly the microviscosity (intracellular viscosity of RBC) decreases. In consequence, the effect of the developed heat of friction on RBC elongation are considered to be negligible.

Uniform and laminar flow within the gap are prerequisites for ektacytometry and laser diffractoscopy. Uniformity is achieved by making the gap width as narrow as possible. However, doing so, the probability of occurrence of turbulences increases. Experience has shown that a gap width of 0.5 mm guarantees rather uniform flow. The occurrence of Reynolds and Taylor turbulences can be calculated using the equations given in figure 7. Taylor rolls, typical for Searle - type viscometers (left part of figure 7), are expected if a number of 41.3 is reached. The usual, non directed turbulence, is reported to be present in Searle- and Couette - systems at Reynolds numbers ³ 400. At a given geometry of the viscometer the probability of occurrence of turbulences increases with decreasing viscosity and increasing shear rate. As shown in the right part of figure 7, viscosity has to be reduced to 2.2 cpoise in order to produce Taylor rolls and to 1.5 cpoise to elicit Reynolds turbulence at the high shear rate of 2600/s. Hence under the usual experimental conditions the occurrence of turbulences can be excluded.

The computer assisted control of motor speed and the position control device allow to determine elongation of RBC at low shear stress with reasonable accuracy. Until now investigation using low shear stress could only be performed by means of reducing the viscosity of the surrounding fluid. Mohandas et al.^{7,5} reported that, in presence of low viscosity (5.2 cpoise), the elongation curve oscillates in the range of low shear stress and suggested that this phenomenon may be based on cell orientation. Since under the experimental conditions chosen, the viscosity was lower than the micro viscosity (intracellular viscosity) the

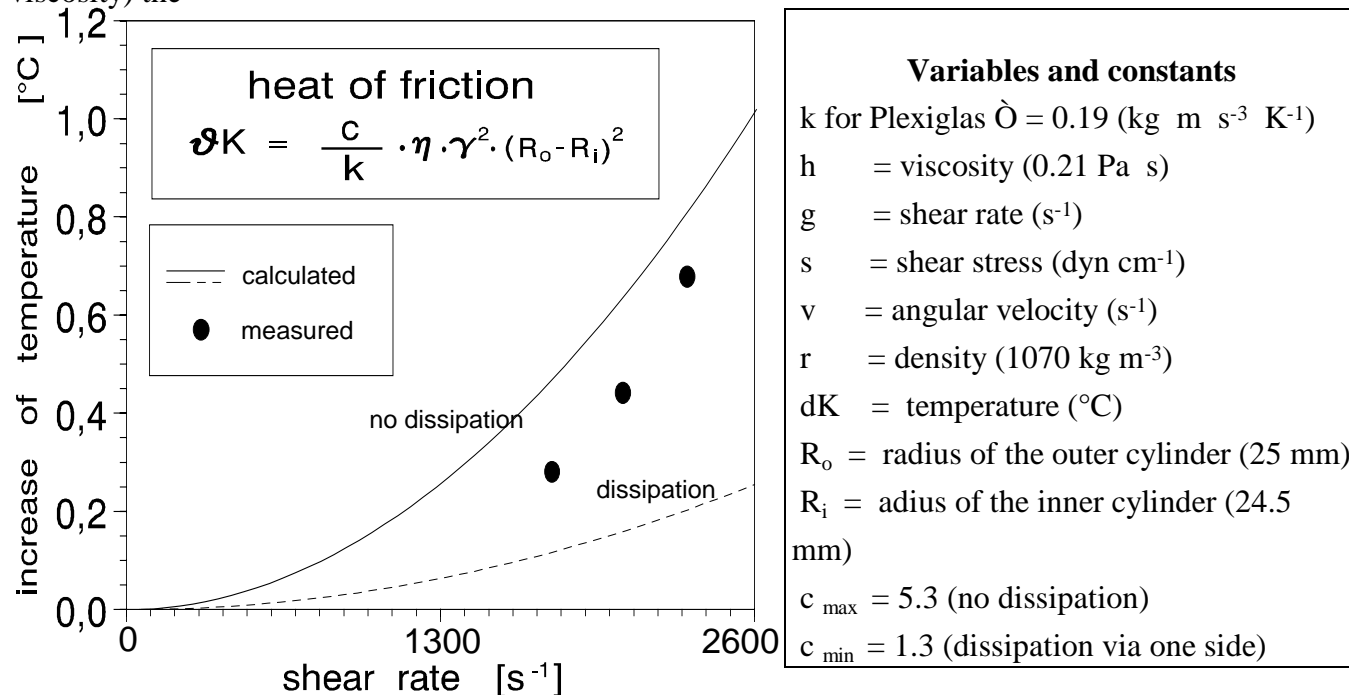


Fig. 6: Increase of temperature due to heat of friction. Calculations assuming no dissipation (solid line) or optimal one-sided dissipation (dashed line).

Taylor number

$$Ta = \frac{\omega \cdot \rho}{\eta} \cdot R_i^2 \cdot \left[\frac{R_o}{R_i} - 1 \right]^{3/2} \geq 41.3$$

Reynolds number

$$Re = \frac{\omega \cdot \rho}{\eta} \cdot R_o^2 \cdot \left[1 - \frac{R_i}{R_o} \right] \geq 400$$

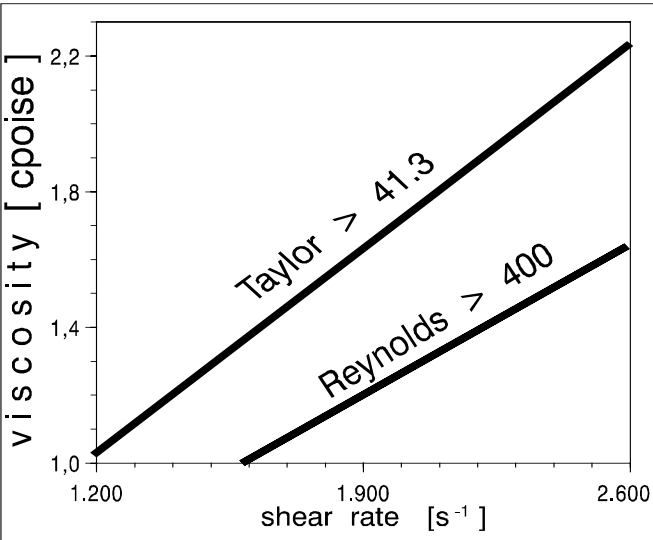
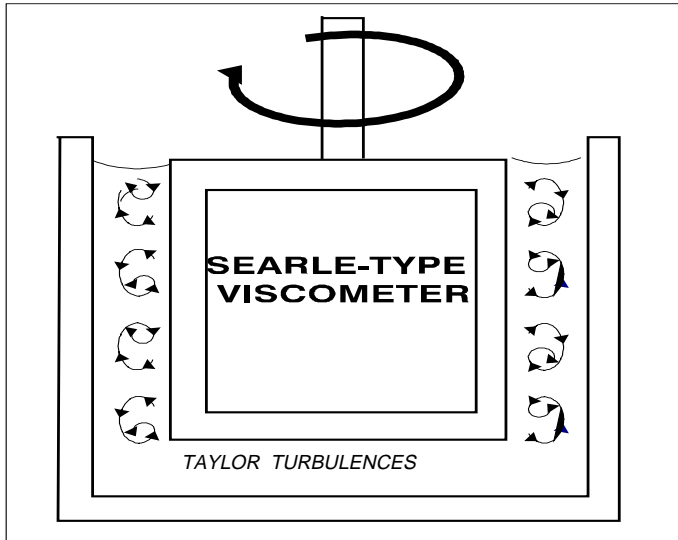


Fig. 7 : Occurrence of turbulences in Searle viscometers as depending on viscosity and shear stress. For explanation of variables and constants see figure 6.

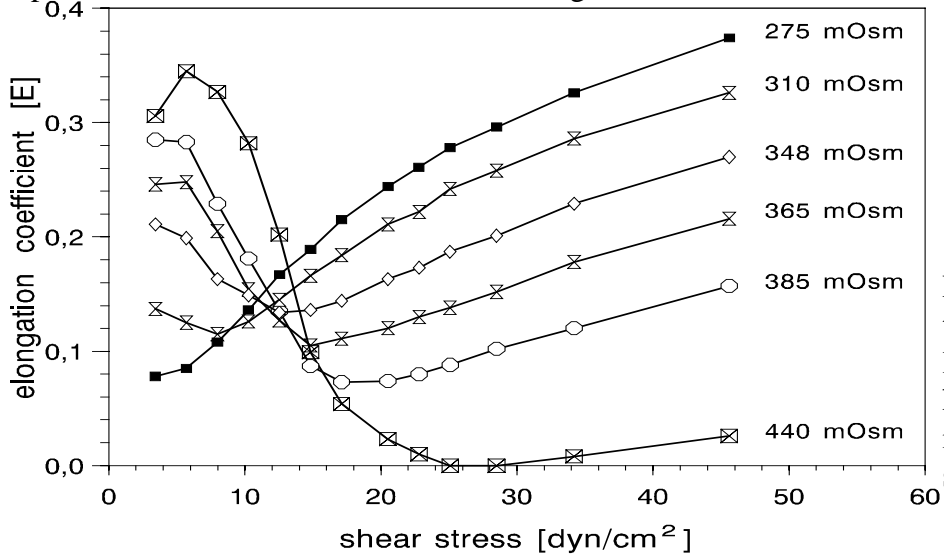


Fig. 8 : Section of the elongation curve corresponding to low shear stress. Note the initial hump, which is accentuated by increasing the osmolarity of the suspending fluid.

the question whether the orientation phenomenon is based on low external viscosity or low shear stress could not be answered.

Figure 8 shows sections of elongation curves corresponding to low shear stress, but determined under the condition of high external viscosity (22 cpoise). It is obvious that there exists an initial hump, which is accentuated by increasing the osmolarity and which disappears in a mild hypotonic solution. We suggest, that at low shear stress the RBC present their small side aspect to the laser beam, such deceiving an elongation. Applying more shear stress the cells turn in such a way that they present their round side,

to the laser beam and then become elongated. In hypotonic solutions RBC become spherical, such that the transition from orientation to elongation is more or less annihilated, whereas in hypertonic solutions more shear stress is needed in order to turn the exsiccated RBC.

In consequence, whenever the flexibility of RBC is characterized by means of measuring elongation in laminar flow, an interference of orientation and elongation should be taken into account.

5. ACKNOWLEDGMENTS

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6. REFERENCES

1. R. Bayer, B. Schauf and B. Günther B, "Erythrocyte shape analysis by means of laser diffraction," SPIE, 1641, pp. 246-255, 1992.
2. R. Bayer and G. Wolf, "Analysis of erythrocyte flexibility by means of laser diffraction : rigidification due to defined shearing," SPIE, 1981, pp. 26-37, 1992
3. M. Bessis, and N. Mohandas, "A diffractomatic method for the measurement of cellular deformability," Blood Cells, 1, pp. 307-313, 1975.(5)
4. M. Bessis, N. Mohandas and C. Feo, "Automated ektacytometry: A new method of measuring red cell deformability and red cell indices," Blood Cells, 6, pp. 315-327 1980.
5. R. Clark, N. Mohandas and S.B. Sohet, "Osmotic gradient ektacytometry : comprehensive characterization of red cell volume and surface maintenance," Blood, 61, pp. 889-910, 1983
6. W. Groner, N. Mohandas and M. Bessis, "New optical technique for measuring erythrocyte deformability with the ektacytometer," Clin. Chem., 26, pp. 1435-1442, 1980.
7. N. Mohandas, R. Clark, M.S. Jacobs and S.B. Sohet, "Analysis of factors regulating erythrocyte deformability," J. Clin. Invest, 66, pp. 563-573, 1980
8. B. Schauf, "Erythrozytenflexibilität," Dissertation, Heinrich- Heine University, Düsseldorf, Med. Fac., 1991.
9. G. Wolf, R. Bayer and D. Ostuni, "Stress-induced rigidification of erythrocytes as determined by laser diffraction and image analysis," Optical Engineering, 31, pp. 1475-1481, 1992
10. T.H. Young, "An introduction to medical literature including a system of practical nosology," Underwood, London, p. 545, 1813