Biochimica et Biophysica Acta, 550 (1979) 288—296

BBA 78240

THE EFFECTS OF SUSPENDING MEDIUM VISCOSITY ON ERYTHROCYTE DEFORMATION AND HAEMOLYSIS IN VITRO

D.R. MORRIS and A.R. WILLIAMS *

Department of Medical Biophysics, University of Manchester, Manchester M13 9PT (U.K.)

(Received May 11th, 1978)

Key words: Deformability; Diffractometry; Viscosity; (Erythrocyte)

Summary

Fresh adult human erythrocytes were suspended in isotonic pH adjusted solutions containing various concentrations of Dextran T.500. The cells were subjected to uniform hydrodynamic shear stress in a Ferranti Shirley Cone and Plate Viscosimeter. The amount of lysis incurred at any given combination of exposure parameters was markedly affected by the viscosity of the suspending medium. Optical diffraction patterns obtained whilst the cells were undergoing shear demonstrated that cellular deformation was also a function of viscosity. Consequently, the distorted shape of the stressed cell must play a crucial role in the haemolytic process.

Introduction

The normal adult human erythrocyte exists in its natural resting state as a biconcave disc of approximately 8.6 μm in diameter and 1.9 μm in thickness [1]. The forces which maintain this shape are the bending forces associated with cellular curvature [2], these are small [3] and consequently erythrocytes deform readily under the application of shear stress.

Erythrocytes suspended in viscous isotonic media distort into long, thin prolate ellipsoids when subjected to laminar hydrodynamic shear fields [4—10, 18]. At low stress values the distorted cells still retain some of their characteristic biconcave shape, indicating that this deformation is isochoric (i.e. is produced without increase in membrane tangential tension or change in cell volume). As the forces of restraint are small, large increments in deformation

* To whom reprint requests should be addressed.

occur for relatively small increments in stress. Upon removal of the stress, the cells return to their biconcave resting state.

At higher stress values, deformations beyond the isochoric limit occur (i.e. the cells no longer have dimples) and the membrane is stretched. The rate of change of deformation with stress is much lower in this region, as there now exist tangential tensions within the membrane which resist cellular deformation. Continued application of stress leads to plastic deformation of the membrane which, if maintained, will result in membrane rupture [11]. The amount of haemolysis incurred depends upon both the magnitude of the stress and the time of exposure to that stress [12]. Upon removal of the stress, the unbroken cells do not return to their original form, instead they take up crenated or asymmetric forms, a clear indication that some sort of structural rearrangement has taken place within the cellular membrane [13].

The human red cell under shear is a dynamic entity, and many different techniques have been utilised in its study. Cine micrographic techniques have been developed [14], and these show that the membrane of the sheared cell 'tank-treads' around the cellular contents. This technique is limited however by the low shear rates required for stability of the cine image. Single tethered cells have been studied [15] and much valuable information has been gained about the physical properties of the cell membrane itself. However, studies of this type do not provide information relating to the dynamic interaction of the cells with the shear field. Other workers have fixed cells whilst under shear and then used scanning electron microscopy techniques to study the deformed cells [8]. This technique provides spectacular results, but again precludes a true dynamic study since diffusion of the fixative to all parts of the cell takes a finite time to occur and, consequently, artifacts may be induced by the tank-tread rotation of the membrane.

As none of the established techniques was considered fully satisfactory for a wide-range dynamic study an alternative technique was developed which allowed us to study the entire population of cells whilst they were actually undergoing shear. A Helium Neon laser beam was passed through the sample and cellular shape and size were determined from the resulting diffraction patterns. The size of the diffraction pattern is inversely related to the size of the cells producing it, e.g.

$$\sin \Theta = 1.22 \frac{\lambda}{D} \tag{1}$$

where Θ is the angle between the laser beam and the first minimum of the pattern (as measured from the sample), λ is the wavelength of the light (6328 Å) and D is the cellular diameter corresponding to that portion of the pattern. Thus, a long thin cell with its long axis alligned in the x direction produces a long thin diffraction pattern aligned in the perpendicular y direction (see Fig. 1). The cells in the shear field align parallel to each other [7] at an angle to the applied field. (A similar effect occurs for deformed oil droplets in a shear field [16].) Thus, the laser diffraction pattern gives us a value of projected length rather than a true length. This technique was developed independently by Bessis and Mohandas [4,5] and our results have confirmed many of their observations.

Method and Materials

Four drops of fresh human blood were obtained by sterile finger puncture from a healthy adult male volunteer and rapidly suspended in viscous isotonic pH buffered Dextran solution (Dextran T.500, Pharmacia, G.B., Ltd.; 300 mosM/l, pH 7.3). An aliquot of the sample (approximately 0.6 ml) was loaded into the Ferranti Shirley Cone and Plate Viscometer and left for 1 min to allow temperature equilibration. The sample was maintained at 25 ± 0.5°C throughout all experiments. Each sample was sheared for 5 min at a single shear stress and was carefully removed using a clean syringe fitted with a (19G2 50/11) needle. 0.2 ml of the recovered sample was pipetted into 2.5 ml of isotonic (300 mosM/l) unbuffered 0.9% saline and centrifuged for 5 min at 3000 rev./min in an M.S.E. bench centrifuge. After centrifugation the supernatant was removed and its absorbance measured at 540 and 460 nm in 1-cm glass cuvettes in a Perkin Elmer spectrophotometer model No. 6/20.

The absorbance values obtained above were compared with the values obtained from four fully lysed samples. Absorbance and percentage haemolysis are linearly related; consequently, a simple ratio gives the haemolysis value at a particular stress. This was repeated at different stresses and the resulting graph of percentage haemolysis as a function of the applied shear stress is called a haemolysis profile (see for example Fig. 2).

During the course of the experiment different viscosity suspending media were required. These were obtained by varying the percentage of Dextran present in solution, great care being taken to ensure that all solutions were isotonic (300 ± 10 mosM/l) and adjusted to pH 7.3. Dextran T.500 has a high molecular weight (500 000) and, consequently, the amounts used in our solutions (up to approximately 15 g/100 ml) contribute little to the osmolarity but does increase the original solvent volume resulting in a net decrease in osmolarity. Consequently, a 15% solution of Dextran 500 was made up in glass distilled water and brought to isotonicity by adding 9 g NaCl per l solution. This solution was adjusted to pH 7.3 with an isotonic alkaline solution of sodium hydroxide. A solution of 0.9% saline was adjusted to pH 7.3 by a similar procedure and was used to dilute the concentrated Dextran solution to the required values. The viscosities of the dilutions were checked using the Ferranti Shirley Viscometer in its normal operating mode.

Optical diffraction patterns are produced when a coherent beam of light is passed through the samples. The coherent beam used in this experiment was produced by a 2 mW HeNe laser (coherent radiation model 80—2 S). A fuller explanation of the formation and interpretation of diffraction patterns can be found in ref. 14. Modifications made to a Ferranti Shirley Cone and Plate Viscometer made it possible for us to obtain patterns from samples actually being subjected to a laminar flow hydrodynamic shear stress field. The modifications entained drilling the plate and inserting a clear perspex plug, and replacing the standard steel cone with a specially produced perspex version. The beam was introduced by means of a high quality plane mirror mounted beneath the plate. The diffraction patterns formed at the sample were projected out of the system by a second mirror mounted above the rotating cone as shown in Fig. 1.

The samples were placed between the cone and plate of the viscometer and,

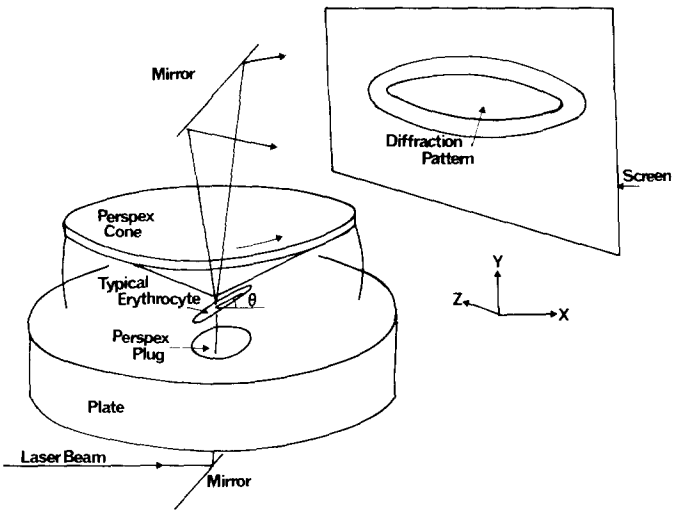

Fig. 1. Schematic illustration of the relative orientation of the cells and the diffraction pattern from them. The screen lies in the YZ plane and the long axis of the pattern is horizontal, i.e. parallel to the Z axis. The erythrocytes lie in the XY plane at an angle Θ to the X direction.

after allowing for temperature equilibration, the cone was run up to the required stress. The elliptical diffraction patterns produced were photographed on 5 × 4 Plus X professional film. After developing, the distortion parameter was calculated from a carefully produced template. The process was repeated with samples of various viscosities.

Results

Fig. 2 presents a selection from the family of haemolysis profiles, obtained when fresh human erythrocytes were disrupted in isotonic pH-adjusted media of different viscosities. Each individual curve retains its sigmoidal shape but it can be seen that both the slope of the linear portion of each curve and the amount of haemoglobin released at any given shear stress increase with increasing viscosity of the suspending medium.

Another series of experiments was performed to obtain the individual haemolysis values at a single shear stress of 1500 dynes · cm^{-2} (shown as a dashed line in Fig. 2) at various viscosities. This shear stress was chosen because fragility curves differ most in this high stress region. Repeated measurements were performed at eight different viscosities in the range 3—20 centipoise, the temperature being held constant at 25 ± 0.5°C. The results are displayed in Fig. 3.

Fig. 3 consists of a central linear region extending from about 6 to 10 cP which subsequently curves towards an asymptotic value at high viscosities. The initial part of this linear portion of the curve undergoes an abrupt transition at about 6 cP. Below this value it appears that the viscosity of the suspending medium has little effect on erythrocyte disruption at this shear stress.

The diffraction patterns presented in Fig. 4 show that the shape of erythrocytes, while they are being subjected to a given shear stress, is dramatically altered in media of different viscosities. Fresh human erythrocytes were sus-

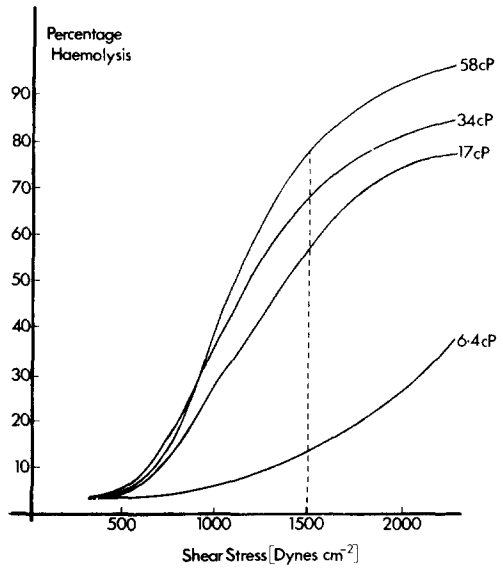

Fig. 2. Haemolysis profiles produced at various suspending medium viscosities.

pended in the same pH-adjusted isotonic media used in the above series of experiments. Laser diffraction patterns were obtained, as described under Method and Materials, at the same uniform hydrodynamic shear stress of 1500 dynes · cm^{-2}.

Fig. 4a was obtained at a viscosity of 3 cP. It clearly shows that the red cells, which give a circular diffraction pattern in their resting state, have undergone minimal distortion despite the high applied shear stress. Fig. 4b depicts an intermediate level of distortion and was obtained at a viscosity of 8 cP. Fig. 4c was obtained at 13 cP and shows that the cells have been distorted to an even greater extent and are in fact approaching the limits of their extensibility. The

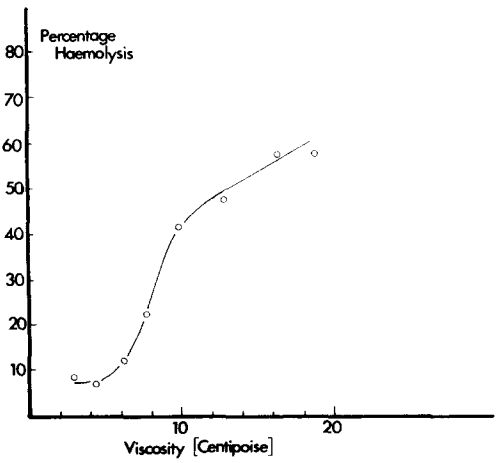

Fig. 3. The variation of haemolysis with suspending medium viscosity at 1500 dynes · cm^{-2}.

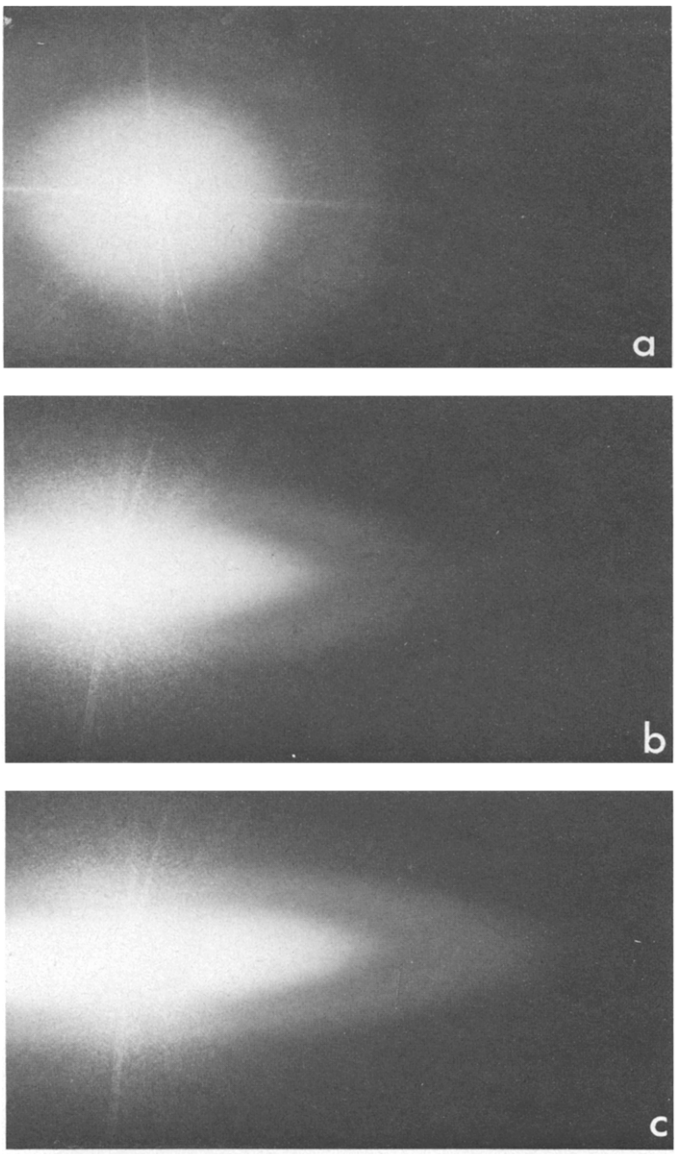

Fig. 4. Diffraction patterns obtained from the system at a constant stress of 1500 dynes, a, 3 cP; b, 8 cP; c, 13 cP.

information concerning the shape of the stressed erythrocytes is contained within the diffraction patterns and may be conveniently expressed in terms of a distortion parameter ϕ defined as $(L - B)/(L + B)$ where L is the length of the diffraction pattern and B its width [16]. The parameter ϕ for this pattern would be exactly equal to a parameter ϕ calculated for the cells if the cells were perpendicular to the beam; however, the cells lie at an angle to the beam and there must exist a discrepancy between the value of ϕ for the pattern and that

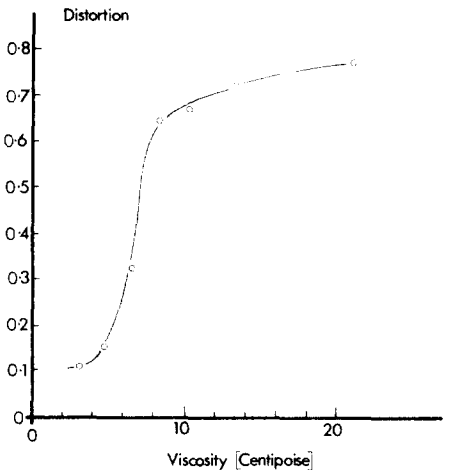

Fig. 5. Graphical representation of the distortion parameter obtained from a complete series of diffraction patterns, at a constant stress of 1500 dynes.

for a cell. The parameter ϕ incorporates measurements of both width and projected length of the cells. Diffraction patterns were obtained at 1500 dynes · cm^{-2} for each of the nine different sample viscosities described in Fig. 3. The distortion parameter derived from each pattern is presented as a function of viscosity in Fig. 5.

Fig. 5 is similar to Fig. 3 in that there is an abrupt discontinuity at about 6 cP and above this value the cellular distortion increases linearly with increasing viscosity. However, a second abrupt discontinuity is observed at about 10 cP, above this value the distortion parameter increases slowly until it attains an asymptotic value of about 0.78.

Discussion

The results presented in Fig. 2 clearly show that the viscosity of the suspending medium affects the haemolytic process. Each individual fragility curve retains its sigmoid shape, reflecting the distribution of mechanical strengths within the normal heterogeneous erythrocyte population. As the viscosity of the suspending medium is increased the linear portion of each curve becomes more steep, accentuating the differences between the curves in the high stress region. From Fig. 2 we see therefore that any given erythrocyte is more easily ruptured at a particular combination of shear stress exposure time and temperature when it is suspended in a medium of increased viscosity. Fig. 3 illustrates the differences in haemolysis at the single stress of 1500 dynes · cm^{-2}.

Fig. 4a shows that erythrocytes subjected to 1500 dynes · cm^{-2} in a 3 cP medium have undergone very little distortion. In Fig. 4c we see a portion of the same cell population subjected to an identical stress, but now suspended in a 13 cP medium. In this latter case the cells are markedly distorted. Clearly, under given exposure conditions the cellular deformation is increased in a medium of

increased viscosity. This confirms the observations reported by Bessis and Mohandas [4,5].

Fig. 5 shows the distortion parameters obtained from a series of diffraction studies as a function of viscosity at the constant shear stress of 1500 dynes · cm^{-2}. Both Figs. 3 and 5 show marked discontinuities in both the 5 cP and the 8—10 cP regions. This result strongly implies that the amount of haemolysis incurred at a given stress depends on the amount of distortion the cell has undergone, i.e. the shape of the cell is a crucial factor in the haemolytic process.

From Fig. 5, we see that under the conditions found in vivo (i.e. in a suspending medium viscosity of approximately 2 cP), the deformation is very small, even though a shear stress of 1500 dynes · cm^{-2} is being applied. Fig. 3 shows that the subsequent haemolysis produced at this deformation is also low and so the red cell in its natural environment (η = 4 cP) is in a state of maximum resilience to shear stress.

Below 5 cP we see little deformation or haemolysis. Above this value, however, both parameters increase dramatically. It would appear that there may exist a critical ratio of internal to external viscosity at which the coupling mechanism of the cells to the suspending medium changes. A similar effect is known to take place for emulsion droplets in suspension [17]. As the viscosity of the suspending medium is increased, both curves undergo another transition in the 8—10 cP region. Here, the cells with the smallest surface area to volume ratio have reached their maximum isochoric deformation (isochoric deformation is limited by the 'excess' surface area a cell possesses, i.e. is determined by its surface area to volume ratio which varies within the population [18]). A value of approximately 0.7 on our deformation scale represents a state of deformation which, for the average cell, accounts for all of its 'excess' surface area. The cells now build up tangential tensions which oppose the distorting forces. This we see as a decrease in the rate at which deformation changes with increasing viscosity and also a consequent decrease in the rate at which haemolysis increases. The two curves cannot be expected to agree exactly since they refer to different parts of the population; Fig. 5 refers to the mean of the entire population, whereas Fig. 3 refers to the varying percentage of cells which have broken at any given shear stress.

In the 5—10 cP region we get appreciable amounts of haemolysis (up to 40%) during the 5 min exposure. Most theories accounting for haemolysis explain it in terms of the tangential tension in the membrane, however, in the 5—10 cP region, the deformations are such that the cells are still deforming isochorically, hence there is little or no tangential tension and yet there is appreciable haemolysis. Again we conclude that the shape of the cell must play a part in haemolysis. The distorted shape of the cell induces bending strains at the tip of the ellipsoidal cell, and it may be that the 'tank-treading' of the membrane over these high bending strain areas causes progressive structural rearrangement within the membrane, which eventually leads to haemolysis. This phenomenon is currently under examination at this establishment, and preliminary results lend support to this 'fatigue' hypothesis.

In the last decade many different techniques have been utilised in the study of erythrocyte deformation and rupture. Estimates of the stress required to

rupture the cells differ from technique to technique. These differences may largely be explained in terms of the different exposure times to which the cells were subjected. However, the above results clearly show that the differing viscosity of the suspending media utilised in some experimental systems could also contribute significantly to the variations between the different techniques.

The above results appear to be independent of the thicknening agent used within the suspending medium, provided of course the solutions are isotonic and adjusted to the same pH. Virtually identical distortion and haemolysis values were obtained when isoviscons solutions were subjected to the same shear stress in the presence of Ficoll, Dextran or polyvinylpyrrolidone.

References

1 Geigy Scientific Tables (1970) (Diem, K. and Lentner, C., eds.)
2 Canham, P.B. (1970) J. Theor. Biol. 26, 61—81
3 Evans, E.A. and Lacelle, P.L. (1975) Blood 45, 29—43
4 Bessis, M. and Mohandas, N. (1975) Blood Cells 1, 307—313
5 Bessis, M. and Mohandas, N. (1975) Blood Cells 1, 315—321
6 Schmid-Schonbein, H. (1975) Blood Cells 1, 285—306
7 Schmid-Schonbein, H. and Wells, R. (1969) Science 165, 288—291
8 Sutera, S., Mehrjardi, M. and Mohandas, N. (1975) Blood Cells 1, 369—374
9 Williams, A.R. and Morris, D.R. (1978) Angiology 29, 53—59
10 Chien, S. (1977) Blood Cells 3, 71—99
11 Evans, E.A. and Hochmuth, R.M. (1976) Biophys. J. 16, 13—26
12 Williams, A.R. (1973) Biorheology 10, 313
13 Williams, A.R. (1973) Biorheology 10, 303
14 Lipson, S.G. and Lipson, H. (1969) Optical Physics, Cambridge University Press
15 Hotchmuth, R.M., Mohandas, N. and Blackshear, P.L. (1973) Biophys. J. 13, 747
16 Taylor, G.I. (1934) Proc. R. Soc. 29, 501
17 Goldsmith, H. and Mason, S.G. (1965) in The Microrheology of Dispersions, (Eirich, F.R., ed.), Rheology, Theory and Application, Vol. 4, Academic Press, New York
18 Canham, P.B. and Burton, A.C. (1968) Circ. Res. 22, 405