SHAPE TRANSFORMATION OF ERYTHROCYTES DETERMINED BY LIGHT SCATTERING CHANGES ASSOCIATED WITH RELAXATION OF PARTICLE ORIENTATION

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ABSTRACT Dilute suspensions of normal erythrocytes exhibit a pearl-like sheen (nacre) when subjected to flow. This results from the asymmetric form (biconcave disc) of these cells. Upon cessation of flow, the nacreous effect decays in 2–3 min, corresponding to the time required for the discs to achieve random orientations by rotatory Brownian motion. The degree of nacre can be measured by comparing the intensity of scattered red light at an angle of 45° for the flowing system to that when the effect has disappeared. Since the phenomenon is critically dependent on red cell shape, it is possible to quantify the extent of erythrocyte disc-to-sphere transformation induced by detergents, low salt concentrations, or metabolic depletion.

INTRODUCTION

When a suspension of nonspherical particles is caused to flow in a velocity gradient the particles will tend to orient with their large dimension in the direction of flow. If the particle exceeds a few microns in its largest dimension simple swirling in a test tube will achieve almost complete orientation. The swirling suspension will take on the aspect of a sheen or pearliness (nacre) due to reflection of light by sheets of the oriented particles. This is readily seen with suspensions of, for example, needle-shaped crystals and, indeed, nacre of flow is frequently used as a criterion that crystallization has taken place. When swirling is ceased the nacre decays because of rotational Brownian diffusion and the asymmetric particles assume random orientations.

Ponder, in his book (ref. 1, p. 40), briefly mentions that a swirling suspension of red cells exhibits a sheen. We have found that when normal human whole blood is diluted approximately 100-fold in isotonic saline (0.9% NaCl) the nacre of flow is particularly apparent. The sheen persists as long as the suspension is swirled. When swirling is ceased the nacre decays and disappears after 2–3 min.

Normal human erythrocytes are biconcave discs about 8 μm in diameter, 2 μm thick at the periphery and 1 μm thick at the narrowest portion. As will be shown, any treatment of the cells which cause them to become less asymmetric decreases the time of persistence of nacre. It is the purpose of the present paper to describe a light scattering method for following the decay of nacre when swirling is ceased and to apply this method for studying changes in shape of red cells.

THEORY

With sufficiently high shear gradients asymmetric particles may be aligned along the flow lines. When the flow is ceased the particles assume random orientation with a rate determined by the rotational diffusion relaxation time, τ, for the particles. The decay of orientation is exponential with time according to the expression.

$$\overline{\cos \alpha} = \exp(-t/\tau), \tag{1}$$

where $\overline{\cos \alpha}$ is the average over all particles of the cosine of the angle which the long axis of the particle makes with the direction of flow. The relaxation time is thus defined as the time to reach $1/e$th of the completely aligned value, namely unity. Perrin (2) has calculated the rotational relaxation time for an oblate spheroid of semi-axis b and a in terms of b and the axial ratio $\rho = b/a$. The complicated expression involves an arc tangent function of the square root of $\rho^2 - 1$. If the particle is highly assymmetric, i.e. the oblate spheroid approaches a flat disc, the smaller dimension, a, disappears and the expression for τ becomes simply

$$\tau = 2\eta d^3/3kT, \tag{2}$$

where T is the absolute temperature, k is Boltzmann's constant, d (equal to 2 b) is the diameter of the disc, and η is the viscosity of the medium. Numerical calculations (3) show that Eq. 2 is within 10% of the complicated Perrin formula if the length of long axis of the particle exceeds that of the shorter axis by a factor of four or more. The normal human erythrocyte is more complicated than an oblate spheroid but, because of the narrowing at its center may act hydrodynamically like a disc with $b > 4a$, and hence Eq. 2 should be valid. Taking $b = 4$ μm and for room temperature where $\eta = 0.01$ poise the relaxation time is calculated to be $\tau = 80$ s.

Erythrocytes, like other particles with dimensions large compared with the wavelength of light, scatter light predominently in the forward directions (4). For suspensions of such large particles at finite concentrations the situation is complicated by multiple scattering. Light scattered by one particle is scattered by another which, in turn, is scattered by another, and so on. To account for multiple scattering requires formidable numerical calculations (4) which would reduce the usefulness of the method. We observed, however, that swirling a dilute suspension of erythrocytes diminishes its gross turbidity relative to that which it had when stationary. When

swirling is stopped the particles assume random orientation by rotary Brownian motion with a consequent increase in light scattering. It was found that for red light the greatest difference in intensity of scattered light between a swirling suspension and a stationary suspension occurs when the scattering is viewed at 45° (angle taken from the transmitted beam).

METHODS AND RESULTS

Light scattering was measured in the Aminco light scattering photometer (5) (Aminco, Silver Spring, Md.) which was modified for our experiments. The mercury vapor lamp was replaced by a 1 mW helium-neon laser (model 132, Spectra-Physics, Inc., Mountain View, Calif.). This provides red light of wavelength 638.2 nm which is not absorbed by hemoglobin. The photomultiplier was set at an angular reading of 45°. The photocurrent was recorded on a Hewlett-Packard type 7101B recorder (Hewlett-Packard Co., Palo Alto, Calif.) operated at a chart speed of ½ in/min.

The light scattering vessels are glass cylindrical vials such as used for scintillation counting (type 3-337-5, Fisher Scientific Co., Pittsburgh, Pa.) with a 22 ml capacity and provided with a plastic cap. A simple base was constructed to insure that the center of the vial was set at the center of rotation of the angular light scattering photometer.

The procedure consists simply of rapidly swirling, in a rotary fashion, by hand the capped cell suspension (20 ml), quickly placing it in the photometer, and recording

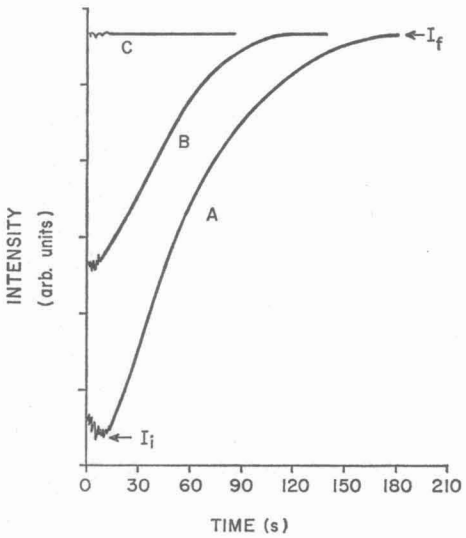

FIGURE 1 Time course of light scattering (638.2 nm wavelength, observation at 45°) for normal blood washed and diluted 1:400 in 0.9 NaCl. Time taken immediately after swirling. (*A*) Control. Sodium dodecyl sulfate, (*B*) final concentration 2 μM, (*C*) final concentration 4 μM.

the time course of the light scattering signal. Because of the long rotational diffusion relaxation time of the particles compared with the relaxation time for the fluid rotary movement, the extent and rapidity of the swirling is not critical. For a vial of the form and size employed (1-in diameter) and for a fluid of the viscosity of water, inertial circulatory motion of the liquid ceases after about 15 s.

The time course of the decay of the nacre of flow after swirling is stopped is shown in Fig. 1, curve A. Initially, the readings are erratic but within a few seconds become smooth and over the course of 3 min reaches the final value I_f. The difference, ΔI, between initial intensity, I_i, and the final value I_f, is reproducible within 2%. A number of whole blood dilutions (in 0.1% saline) were made from 1:100 to 1:1000 (50-5 million red cells/ml, respectively) and it was found that both I_f and ΔI are proportional to the concentration of the erythrocyte concentration. Hence, $\Delta I/I_f$ is independent of concentration. For a given erythrocyte suspension ΔI is greatest when the observation is at 45° off axis while at 20° it is zero. Below 20° ΔI is actually negative. This is in agreement with Kuroda et al. (6), who found that the transmission of red light through erythrocytes suspensions increases when the system is caused to flow.

In Fig. 1 is also shown the effect of adding increasing amounts of the anionic detergent, sodium dodecyl sulphate. The value of ΔI diminishes linearly with detergent concentration up to 3 μM.

Defibrinated whole blood was diluted 1:400 in phosphate-buffered (pH 7.4) saline solutions of various concentrations. As seen in Fig. 2 with decreasing concentrations of NaCl from 0.9% to 0.5% ΔI decreases in a linear fashion. Below 0.5% the erythrocytes begin to lyse and I_f falls.

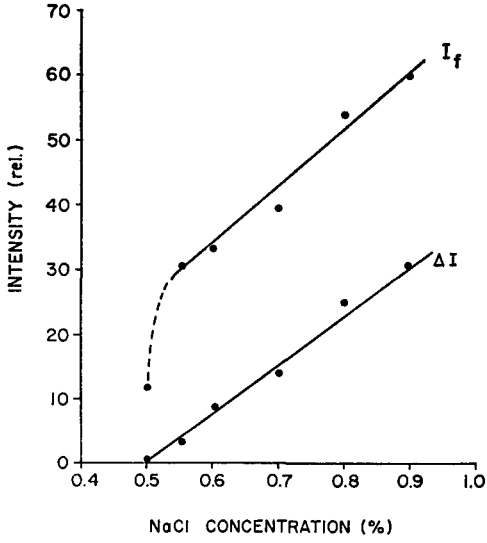

FIGURE 2 ΔI and I_f for blood diluted 1:400 as a function of salt concentration, pH 7.4.

Erythrocytes were treated with hypotonic saline or with detergent so as to give a zero value of ΔI. These cells were then fixed with buffered formaldehyde (2% final concentration), then washed and resuspended in isotonic saline. The resuspended cells again showed ΔI equal to zero. Increasing proportions of these fixed cells were added to suspensions of untreated erythrocytes in isotonic saline, maintaining the final total cell concentration constant. It was found that ΔI is proportional to the fraction of untreated erythrocytes.

An erythrocyte suspension without supplemental glucose was incubated at 37°C. A noticeable reduction in ΔI occurs after 4–6 h of incubation. After 20 h of incubation ΔI is zero but I_f remained the same as the value obtained at the start of incubation.

DISCUSSION

Since the relaxation of orientation of asymmetric particles according to Eq. 1 is exponential in time, a test of the method (and/or applicability of the theory) is to see if the logarithm of ΔI is linear in time. Using the data of Fig. 1 A it is found that $\log \Delta I$ is proportional to time and yields a relaxation time $\tau = 65$ s. This is smaller than the value calculated from Eq. 2. Erythrocytes are not rigid structures. Under the phase microscope if the red blood cells are caused to flow by gently pressing on the cover slip one sees that the cells tumble with a wobbly motion. This flexibility would account for the observed lower relaxation time.

The extent of the nacre of flow given by ΔI is clearly a function of the shape of the erythrocytes. Both the detergent and the hypotonic saline solutions cause a rounding of the cells. Indeed, the fixed treated cells which we described appear spherical in the phase microscope. Intermediate concentrations of the detergent can cause crenation (ref. 1, chapter III), but, in any case, the cells become less asymmetric than normal cells which provides a reasonable explanation for the decrease in nacre.

Addition of the detergent to erythrocytes causes a sphering of the cells without changing their volume (ref. 1, chapter II). This is born out in Fig. 1, curves A and B, where the detergent does not alter I_f. On the other hand, when erythrocytes are suspended in hypotonic salt solutions they behave as osmometers and undergo swelling with an increase in volume (ref. 1, chapter III). This is manifested by a decline in I_f with decreasing salt concentration (Fig. 2). Unlike disc-to-sphere transformation which occurs without volume change, hypotonic swelling of erythrocytes results in a lowering of I_f with a slope close to that for ΔI. This lowering of intensity of light scattering probably results from the decrease in refractive index of the cells (essentially by dilution of hemoglobin) as they imbibe water on swelling and, hence, is an indication of volume change. When lysis occurs at salt concentrations below about 0.5% I_f drops because the cell ghosts have a refractive index close to that of the aqueous medium. Our method thus permits a sensitive measure of minimal degrees of cell swelling in the prehemolytic phase. The fact that both ΔI and I_f

decline linearly with lowering salt concentration shows that osmotic swelling is being measured directly.

Incubation of erythrocytes at 37°C without supplemental glucose is known to result in metabolic depletion with loss in cell deformability (7). The reduction in ΔI which we observed within 4–6 h of incubation coincides with the detectable increase in red cell rigidity as measured by the microcapillary technique for cells treated in this manner. The fact that throughout 20 h of incubation I_f remained constant indicates that the treatment caused no change in cell volume but the cells became spherical since the nacre disappeared. For finite values of ΔI, however, our method in its present form does not distinguish between a uniform tendency toward sphering and a mixture of totally sphered and normal cells.

The use of the light scattering technique for the estimation of red cell shape offers advantages over such methods as direct microscopic visualization, micropipetting (7), and filtration through micropores (8), all of which may introduce artifacts caused by cell contact with solid surfaces. Our method is simple, sensitive, reproducible, and gives an average value over a large population of cells as opposed to the single cell measurements.

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