Comparison between Internal Microviscosity of Low-Density Erythrocytes and the Microviscosity of Hemoglobin Solutions: An Electron Paramagnetic Resonance Study

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ABSTRACT The hypothesis that the internal viscosity of erythrocytes is governed by the intracellular hemoglobin (Hb) concentration is examined. Here viscosity is determined by labeling of the cytoplasmic reduced glutathione with the spin label maleimido-Tempo. Erythrocyte populations with different Hb concentrations in isosmotic conditions were obtained through incomplete lysis, followed by cell resealing, and discontinuous density gradient separation. This procedure maintains normal cell shape and volume. Microviscosity of membrane-free Hb solutions was measured by addition of spin labeled glutathione. It was found that microviscosity values are similar for the erythrocyte cytoplasm and for Hb solutions of equivalent concentrations, showing that the erythrocyte membrane does not have any influence on internal microviscosity. The dependence of the microviscosity on the concentration of Hb solutions was compared with results of macroscopic viscosity obtained by other authors. It is concluded that microviscosity is sensitive to individual properties of the Hb molecule (intrinsic viscosity), but that it is not sensitive to intermolecular interactions. As the microviscosity behavior as a function of Hb concentration is the same in Hb solutions as in the erythrocyte cytoplasm, the inferences regarding macroscopic viscosity in Hb solutions could be translated to the rheological properties of the erythrocyte cytoplasm. Thus, these properties could be predicted from the values of the mean corpuscular Hb concentration.

INTRODUCTION

Rheological properties of blood are governed by several factors. Among them, rheological properties of erythrocytes, which comprise viscoelastic properties of the erythrocyte membrane and viscosity of the internal medium, are of crucial importance (Dintenfass, 1971). A mature erythrocyte is devoid of organelles, and it can be considered to be a membranous bag of hemoglobin (Hb) (Voet and Voet, 1990). Consequently, the internal viscosity of normal erythrocytes should be dependent only on the Hb concentration.

Electron paramagnetic resonance (EPR) and nuclear magnetic resonance (NMR) spectroscopies can be applied to measure correlation times of suitable labels introduced into the erythrocyte cytoplasm, leading to the evaluation of the local viscosity ("microviscosity" (Tanford, 1980)). This magnitude is related to the resistance to motion in a molecular scale, whereas "macroscopic viscosity," i.e., that measured with conventional viscometers, is related to the energy dissipated in maintaining a stationary flow of the substance under study (Tanford, 1961).

The dependence of cytoplasmic microviscosity on Hb concentration was investigated in several EPR and NMR studies (Morse et al., 1979; Daveloose et al., 1983; Herrmann and Muller, 1986; Endre and Kuchel, 1986). In all of them, Hb concentration was modified by suspension of the erythrocytes in media of varying osmolality. Morse et al. (1979), and Morse and Warth (1990) reported an enhanced microviscosity inside the erythrocyte cytoplasm, compared with that in Hb solutions of similar concentrations. This fact was attributed to cytoplasm-membrane interactions. However, EPR experiments performed by Herrmann and Muller (1986) in erythrocytes and Hb solutions allowed them to conclude that the cell membrane does not influence internal microviscosity. They remarked on the differences between the microviscosity and the macroscopic viscosity of Hb solutions. In a NMR study, Endre and Kuchel (1986) also analyzed these differences. They found microviscosity values lower than the corresponding macroscopic viscosities, and attributed them to the differences in size between the probe and the solute molecules.

The purpose of the present research is to investigate EPR cytoplasmic microviscosity of erythrocytes, and microviscosity of Hb solutions of similar concentrations, and to interpret the results in comparison with those for macroscopic viscosity of Hb solutions from several authors. Normocytic erythrocytes with variable Hb concentration near and below physiological values were obtained in isosmotic conditions (Luquita et al., 1996). The addition of a spin label that permeates the erythrocyte membrane, and binds covalently to the reduced glutathione present in the cytoplasm, allowed us to obtain the EPR spectra, from which correlation times and microviscosity values for each Hb concentration were evaluated. EPR microviscosities of membrane-free Hb solutions were measured by addition of labeled glutathione. Thus, the same labeled molecule measures microviscosity in both systems.

Received for publication 15 June 1995 and in final form 26 March 1996.
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0006-3495/96/07/389/05 $2.00

MATERIALS AND METHODS

Blood samples

20 ml of whole blood were obtained from healthy donors; Heparin was used as the anticoagulant. Whole blood was centrifuged at 2000 × *g* for 10 min, and a portion of the plasma was reserved for later use. Erythrocytes were washed in isotonic phosphate buffered saline (PBS, 150 mM NaCl, 5 mM KH_2PO_4 + Na_2HPO_4 with 2 mg/ml glucose, pH 7.4, 310 mOsm/kg), and resuspended in the same solution at an 80–90% hematocrit.

Incomplete lysis and resealing

This method is based on typical osmotic lysis procedures (Schwoch and Passow, 1973; Nash and Meiselman, 1983). In this case, the lysis is incomplete because of the high concentration of the erythrocyte suspension, and the brief exposure to hypotonic conditions at 0°C (Luquita et al., 1996).

4 ml of 0°C diluted phosphate buffer (10 mM KH_2PO_4 + Na_2HPO_4, pH 7.4) containing 4 mM $MgSO_4$, and 0.5 mg/ml ATP were added to an equal volume of resuspended ice-cold erythrocyte suspension at 80–90% hematocrit. After 5 min at 0°C, isotonicity was restored by the addition of 0.96 ml of resealing solution (480 mM KCl, 120 mM KH_2PO_4 + Na_2HPO_4, with 8 mg/ml glucose, and 5 mM reduced glutathione (Sigma Chemical Co., St. Louis, MO). Resealing was promoted by 1 h incubation at 37°C. The resealed cells were centrifuged at 20,000 × *g* for 10 min, and then washed in excess PBS after removal of the supernatant. These procedures allowed us to obtain resealed normocytic erythrocytes with a distribution of values of mean cell Hb concentration (MCHC) between 15 and 36 g/dl. The next step was their separation by means of a discontinuous density gradient.

Density gradient

Dextran with a mean molecular weight 40,000 (DX 40, Sigma) was dispersed in distilled water to specific gravities 1.06, 1.07, and 1.08 at 20°C (measured by refractometry and picnometry). The osmolality was measured with a vapor pressure osmometer (Wescor Inc., Logan, Utah), and adjusted to 300 mOsm/kg with dry NaCl. The three solutions were layered into a cellulose nitrate tube to constitute the density gradient (Mach and Lacko, 1968), and packed resealed erythrocytes were deposited on top of the gradient. The tubes were spun at 51,800 × *g* for 48 min at 20°C in an ultracentrifuge (Presvac, Buenos Aires, Argentina). Each erythrocyte layer was removed by gentle suction, washed three times in PBS, and then resuspended in the autologous plasma at a 40% hematocrit. In this way, cell populations with MCHC in the ranges 15–25 g/dl (1.06 layer), 26–32 g/dl (1.07 layer), and 33–36 g/dl (1.08 layer) were separated. The biconcave discocyte normal shape of resealed cells was checked by optical microscope observation. MCHC for each layer was measured as described below.

Biochemical determinations

Hb concentrations were determined spectrophotometrically by the cyanmethemoglobin method (Wintrobe, 1981) at 540 nm, both for Hb solutions and for resealed erythrocyte solutions. Packed cell volume of erythrocyte solutions was measured by the microhematocrit method. MCHC was determined from Hb concentrations and the corresponding packed cell volumes. Membrane phospholipids were extracted, and their phosphorus content was determined by the method used by Sutera et al. (1985). The constancy of this parameter showed the preservation of cell surface after the lysis and resealing procedures.

Preparation of hemoglobin solutions

Erythrocyte lysis without membrane fragmentation was promoted by sonication of densely packed cells for 15 s with a 50 W sonifier (MSE, London, England) (Herrmann and Muller, 1986). The suspension was then centrifuged for 1 h at 45,000 rpm and 10°C in an L8–80 M ultracentrifuge (Beckman Instruments, Inc., Palo Alto, CA, rotor type 80 Ti). The supernatant, which was free from cell debris, was carefully removed. By vacuum evaporation a maximum Hb concentration of 38 g/dl was obtained. Several fractions were further diluted in PBS.

Spin labeling of resealed erythrocytes

Washed resealed erythrocytes from each population were resuspended in PBS at a 10% hematocrit, and 5 μl of a 20 mM ethanol solution of the spin label 4-maleimido-2,2′,6,6′-tetramethyl-piperidine-oxyl (mal-Tempo, Sigma) were added to 3 ml of this suspension under gentle stirring at room temperature. The suspension was allowed to incubate 15 min, and then was washed twice in PBS. No EPR signal was detectable in the supernatant after the second wash. It is well known (Daveloose et al., 1983) that the erythrocyte membrane is permeable to maleimido nitroxides which bind covalently to free thiol groups. For short incubation times, the main binding site is the thiol group of the cytoplasmic reduced glutathione, and the erythrocyte membrane is not permeable to the reaction product (Daveloose et al., 1983; Eriksson and Beving, 1993). Thus, the labeled species that measures cytoplasmic microviscosity is mal-Tempo-glutathione.

Spin labeling of hemoglobin solutions

Equimolar ethanol solutions of reduced glutathione (Sigma) and mal-Tempo were incubated for 15 min. The reaction product (mal-Tempo-glutathione) was added to 3 ml samples of each Hb concentration. The final concentration of labeled molecules was always less than 0.5 mM to avoid concentration-dependent EPR line broadening (Morse at al., 1975).

Electron paramagnetic resonance (EPR) experiments

EPR spectra of labeled erythrocytes and Hb solutions were recorded at 25°C and 9.7 GHz (X band) in a ER-200 spectrometer (Bruker Analytische Messtechnik GMBH, Karlsruhe, Germany). A quartz flat cell was used, and the temperature was controlled to ± 0.5°C. All spectra showed narrow equally-spaced lines, indicative of a highly mobile species in a low viscosity regime (Wertz and Bolton, 1972), and eliminating the possibility of label binding to the cell membrane proteins, or to Hb molecules. The rotational correlation time τ_c (in seconds) of the spin probe was calculated according to the standard formula for isotropic high mobility regime measurements at X band (Raison et al., 1971; Daveloose et al., 1983) as:

$$\tau_c = 6.5 \times 10^{-10} \Delta H_0 [(h_0/h_{-1})^{1/2} - 1], \qquad (1)$$

where ΔH_0 is the peak-to-peak linewidth of the central line of the EPR spectrum (in gauss), and h_0 and h_{-1} are the peak amplitudes of the central and high-field lines. The numerical factor depends both on the magnetic field, and on the anisotropies of the g factor and the hyperfine interaction (Kivelson, 1972). The value 6.5×10^{-10} is customarily used for X band experiments (Raison et al., 1971; Daveloose et al., 1983). As the exact value for mal-Tempo is not known, correlation times calculated with Eq. (1) may be affected by an unknown systematic error. However, the calibration procedure that was used to determine viscosity values ensures that they are free from this error.

We established a calibration curve of τ_c as a function of viscosity by recording the spectra of mal-Tempo-glutathione in sucrose solutions of known concentrations. A straight line ($r = 0.994$, $N = 13$, $p = 9 \times 10^{-12}$) was obtained in the measured range of sucrose viscosities (1 - 9 mPa s). Linear regression yielded a slope of $(8.10 \pm 0.13) \times 10^{-8}$ s (Pa s)$^{-1}$ (T = 25°C). To consider residual line broadening owing to unresolved proton hyperfine structure, we applied the corrections of Bales (1989). The corrected correlation times were 10% lower than those given by Eq. 1. We

obtained microviscosity values from the corrected τ_c values using the calibration curve.

As erythrocyte samples are inhomogeneous, i.e., they contain a large number of cells, with a distribution of values of Hb concentration, a distribution of correlation times around a mean value is expected. Thus, the validity of Eq. 1 is not guaranteed for such a system. To clarify this point, we performed simulations of the central and high-field lines of the EPR spectra. Two unit area Lorentzian functions having linewidths proportional to the correlation time, according to Kivelson's equations (Kivelson, 1972), were convoluted with a Gaussian distribution of correlation times (with mean value τ_0, and rms σ_τ). The correlation time of the simulated spectrum was calculated from Eq. 1, and then compared with τ_0. Agreement within 10% was obtained for the distributions verifying that $\sigma_\tau/\tau_0 < 0.20$. Using the measured τ_c values of Hb solutions, and considering the range of Hb concentrations within each erythrocyte layer, we estimated a maximum $\sigma_\tau/\tau_0 \approx 0.17$. Thus, these considerations show that Eq. 1 gives the mean τ_c values of erythrocyte populations within an error less than 10%. A more realistic model, including the inhomogeneous linewidth that is due to unresolved hyperfine interactions, would yield a better agreement, indicating that it is safe to use Eq. 1 to calculate correlation times from erythrocyte spectra.

RESULTS

Fig. 1 displays the values of erythrocyte cytoplasmic microviscosity, and the values of the microviscosity of Hb solutions plotted as a function of Hb concentration c, which should be interpreted as mean corpuscular hemoglobin concentration in the case of erythrocytes. Erythrocyte microviscosity values at physiological levels of Hb concentration (33 g/dl) are similar to those obtained by other authors (Morse et al., 1979; Daveloose et al., 1983; Herrmann and Muller, 1986).

It can be seen from Fig. 1 that microviscosity data from Hb solutions overlap the erythrocyte microviscosity data, yielding a single function of Hb concentration, which deviates from linear behavior. A least-squares fit was performed to an exponential function,

$$\eta_\mu = \alpha \exp(\beta c), \tag{2}$$

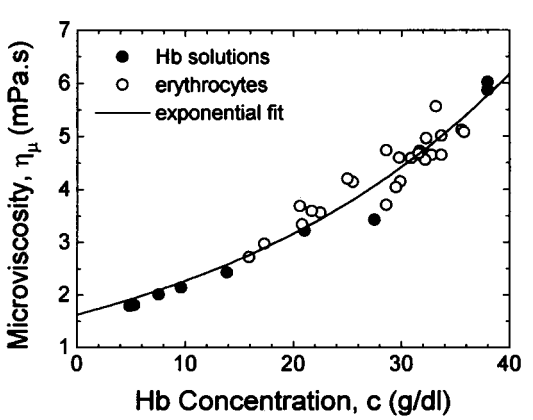

FIGURE 1 Joint plot of microviscosity of hemoglobin solutions (●) and erythrocyte internal microviscosity (○) as a single function of hemoglobin concentration c. For erythrocytes, c should be understood as mean corpuscular hemoglobin concentration. The solid curve is a least-squares fit to an exponential function (Eq. 2).

where η_μ stands for microviscosity. The parameters giving the best fit were $\alpha = (1.61 \pm 0.09)$ mPa s and $\beta = (0.034 \pm 0.002)$ dl/g, where the uncertainties are standard deviations. The value for the exponent β compares well with those of the equations of Herrmann and Muller (1986), describing the concentration dependence of the correlation time of the spin label Tempone in erythrocytes and in Hb solutions. It also reproduces well the concentration dependence of the correlation time of Tempone in solutions of oxyHb A, deoxyHb A, and deoxyHb S reported by Beaudoin and Mizukami (1978)

DISCUSSION

Comparison between macroscopic viscosity and microviscosity of hemoglobin solutions

Our experimental results allowed us to fit a single exponential function (Eq. 2) for the concentration dependence of the microviscosity of erythrocytes and Hb solutions, indicating that Hb solutions are adequate models for the erythrocyte cytoplasm. To interpret this exponential behavior, we shall compare Eq. 2 with the equation describing the dependence on concentration of the macroscopic viscosity of Hb solutions. Hb is a globular protein, and for a solution of quasi-spherical macromolecules the viscosity behavior can be described by the generalized Mooney equation (Ross and Minton, 1977):

$$\eta = \eta_0 \exp\left(\frac{[\eta]c}{1 - \kappa[\eta]c}\right), \tag{3}$$

where η_0 is the solvent viscosity, $[\eta]$ is the macromolecule intrinsic viscosity (related to molecular shape and solvation state), c is the mass concentration of macromolecules, and κ, proportional to the "crowding factor" (Ross and Minton, 1977), is related to intermolecular interactions. The intrinsic viscosities of globular proteins, in aqueous salt solutions at isosmotic conditions, were tabulated by Tanford (1961), and for Hb the intrinsic viscosity is $[\eta] = 0.036$ dl/g. Experimental results for macroscopic viscosity of Hb solutions obtained by several authors with different conventional viscometers are very well reproduced by Eq. 3, using Tanford's value for $[\eta]$, together with an interaction parameter $\kappa = 0.014$ (Cokelet and Meiselman, 1968; Ross and Minton, 1977). In this way, it can be seen that macroscopic viscosity depends on the characteristics of the individual macromolecules, as well as on the interactions among them.

Comparing Eqs. 2 and 3, one can see that Eq. 3, describing the dependence of macroscopic viscosity on Hb concentration, reduces to an equation similar to that governing microviscosity (Eq. 2) in the case of $\kappa = 0$, which means negligible intermolecular interactions. In this way, it may be considered that the EPR microviscosity of erythrocytes and Hb solutions can also be described with a generalized Mooney equation, where the corresponding values are $\kappa = 0$, $\eta_0 = \alpha = (1.61 \pm 0.09)$ mPa s, and $[\eta] = \beta = (0.034 \pm 0.002)$ dl/g. In this

comparison, the solvent microviscosity η_0 is higher than the value (1.2 ± 0.2) mPa s measured by us in labeled PBS, but there is a good agreement with the value of $[\eta]$ given by Tanford (1961). Thus, although microviscosity is related to rotational restrictions imposed on a small labeled molecule, its dependence on concentration carries information about the characteristics of the dissolved macromolecules. The result $\kappa = 0$ obtained for microviscosity data means that the spin label is not sensitive to interactions between Hb molecules, at least for concentrations up to 38 g/dl, the maximum values reached in this work. Herrmann and Muller (1986), and Endre and Kuchel (1986) also remarked on the differences between macroscopic viscosity and microviscosity of Hb solutions, but they did not relate microviscosity to the Hb intrinsic viscosity.

Erythrocyte cytoplasmic microviscosity and the microviscosity of hemoglobin solutions

We have shown that cytoplasmic microviscosity of erythrocytes and the microviscosity of Hb solutions can be described by a single exponential curve (Fig. 1). This fact allows us to conclude that the interactions that restrict the rotation of the labeled molecules in the erythrocyte cytoplasm are the same as those present in a Hb solution of similar concentration. Within experimental error, there is no evidence of increased internal microviscosity resulting from the influence of the erythrocyte membrane. These conclusions agree with those of Herrmann and Muller (1986). However, their results may be a subject of controversy (Morse and Warth, 1990) because Hb concentration in erythrocytes was not measured, but was estimated from the medium's osmolality and pH.

Our results, which we obtained by using the same spin label in the same experimental conditions, and measuring directly Hb concentrations in all cases, show beyond doubt that, with respect to microviscosity, the cytoplasm of low and normal density erythrocytes behaves like a Hb solution of equivalent concentration, and there is no evidence of the influence of any part of the cell membrane on microviscosity. The precision of our experimental data is increased because erythrocytes are selected by a density gradient, which narrows the dispersion of values of Hb concentration around the mean value determined by spectroscopic methods.

Application to blood rheology

Endre and Kuchel (1986) have emphasized that microviscosity results are useful in the study of microscopic processes, but are not adequate for evaluation of energy dissipation in the cytoplasm, when erythrocytes are submitted to deformation and tank-treading motion in blood flow. In the latter case, macroscopic viscosity should be the useful parameter. Inasmuch as we have demonstrated here that, with respect to microviscosity, low- and normal-density erythrocyte cytoplasm behaves as a Hb solution of similar concentration, and it is governed by Hb intrinsic viscosity, we can assume that the macroscopic behavior of both systems will also be similar. Thus, it is reasonable to consider that the rheological behavior of the cytoplasm, i.e., the energy dissipation when the erythrocyte is subject to flow deformation, will be governed by the macroscopic viscosity values calculated by Eq. 3 at the corresponding mean corpuscular Hb concentration.

The validity of these assumptions is limited to normal-shaped erythrocytes with a mean corpuscular Hb concentration at physiological levels or lower, and it is not clear that Eqs. 2 and 3 would be valid for describing cytoplasmic behavior in erythrocytes with higher values of Hb concentration, altered shapes, or both.

This research was supported by the Consejo Nacional de Investigaciones Cientificas y Técnicas (CONICET, grant PID 3761/92), by the Universidad Nacional del Litoral (grant CAI+D 291), and by the Consejo de Investigaciones (CIUR), Universidad Nacional de Rosario. We thank Dr. R. Rigatuso and Dr. A. Rubiolo for allowing us the use of experimental facilities at their laboratories, and Dr. R. Calvo for a critical reading of the manuscript.

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