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# TEMPERATURE JUMP RELAXATIONS IN AQUEOUS SALINE SUSPENSIONS OF HUMAN ERYTHROCYTES

JEFFREY D. OWEN, BRUCE C. BENNION, L. PAULL HOLMES, EDWARD M. EYRING, MICHAEL W. BERG* AND JAMES L. LORDS*

*Department of Chemistry, University of Utah, and Department of Biology*, University of Utah, Salt Lake City, Utah 84112 (U.S.A.)*

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SUMMARY

The Joule heating temperature jump relaxation method utilizing scattered white light intensity detection has been found suitable for measuring kinetic properties of whole cells suspended in aqueous saline solutions. Human erythrocytes suspended in aqueous 0.9% NaCl and subjected to a rapid 8° temperature jump to 25° yield two relaxations: $\tau_1$ of approx. 0.9 msec and $\tau_2$ of approx. 120 msec. The latter appears to be characteristic of intact cells only. On the other hand, $\tau_1$ is obtained with ghosts as well as with whole erythrocytes. Similar experiments were carried out at several cell dilutions, at different scattering angles, at several NaCl concentrations, with other salts, in $^2H_2O$, and with added sodium lauryl sulfate or 1,5-difluoro-2,4-dinitrobenzene. The 0.9-msec relaxation appears to be the time constant for passage of water through the erythrocyte membrane. The principal obstacle to a firmer attribution of $\tau_1$ is the unknown time constant for temperature equilibration of the erythrocyte interior with the heated saline solution.

## INTRODUCTION

In 1935, PARPART[1] devised an opacimetric method for comparing the rate of hemolysis of mammalian erythrocytes in hypotonic aqueous and in hypotonic 99.5% $^2H_2O$ suspensions. He used rapid pneumatic mixing and detected changes in transmitted light intensity with a photocell–torsion string galvanometer combination. He estimated the minimum response time of his apparatus to be 0.05 sec and reported that 99.5% $^2H_2O$ penetrates erythrocytes 44% more slowly than does water.

More recently SHA'AFI *et al.*[2] have used a stopped flow apparatus with a dead time of 11.3 msec to measure rates of human erythrocyte shrinkage in hyperosmolar solution. One syringe is filled with a stock erythrocyte suspension in an isosmolar buffer, and the other syringe contains the same buffer to which NaCl has been added to make it hyperosmolar. The volume of erythrocyte is measured as a function of time by the intensity of white light scattered at 90° to the incident beam. One of their conclu-

*Two whom requests for reprints should be addressed.

sions is that water diffuses through the erythrocyte cell membrane with an approx. 7-msec half time[3].

The Joule heating temperature jump relaxation method of EIGEN AND DE MAEYER[4] is routinely used to study reactions in liquids having half reaction times shorter than the usual shortest time of about 1 msec accessible to stopped flow instruments. Ordinarily light absorption detection is utilized in these temperature jump experiments, and the shortest measurable chemical relaxation times are of the order of 5.0 $\mu$sec. We discuss below Joule heating temperature jump rate studies of isosmolar erythrocyte suspensions in which relaxations of approx. 0.9 msec and approx. 120 msec have been detected in the intensity of white light scattered at 90° to the incident beam.

## METHODS

All the experiments were performed within 4 h of obtaining blood by venipuncture from three different healthy human male donors. The erythrocytes were separated from the heparinized blood by centrifuging the solution for 15 min at 5000 rev./min in a refrigerated Sorvall RC2-B centrifuge. These erythrocytes were then washed, centrifuged, and unless otherwise specified were suspended in 0.9 % NaCl solution (the mammalian isotonic saline solution concentration). Most sample solutions were a dilution of 1/500 from the original concentration of erythrocytes in the body. Thus our temperature jump samples contained approx. $1 \cdot 10^7$ erythrocytes per ml.

Our Joule heating temperature jump apparatus closely resembles that of HAMMES AND FASELLA[5] modified for single-beam operation[6] except for the following features: the light source is an Illumination Industries Inc. Type 110 mercury arc lamp rather than a tungsten lamp. No monochromator is used because it would severely limit the scattered light intensity. The lucite sample cell has 0.5-cm diameter cylindrical light paths of lucite entering and exiting at 90° to one another. An additional 2-cm lucite rod carries the scattered light to the glass envelope of the shielded IP28 photomultiplier tube. This modified temperature jump apparatus has been used previously to measure the rates of surfactant micelle dissociations[7].

Discharging a 0.1-$\mu$F 30-kV capacitor through the sample cell raises the temperature of approx. 1 ml of our sample liquid by approx. 8° in approx. 5 $\mu$sec as verified by light absorbance measurements with methyl orange[8]. The initial sample solution temperature was 17° and the reported relaxation times are for 25 ± 1°. Precipitation of erythrocytes on a msec time scale charactaristic of these experiments is negligible. Blank solutions of aqueous 0.9 % NaCl were run previous to each experiment to verify the absence of detectable relaxations in the scattered light intensity. The sample relaxation times were determined from the exponential oscilloscope traces using semilogarithmic plots of the relative amount of light scattered *versus* the time. The precision of the experimental relaxation times is approx. ± 15 %.

## RESULTS

Our measured relaxation times are shown in Table I. Those of the order of 0.9 msec in the column marked $\tau_1$ are particularly interesting because they are too short to be measured by a stopped flow apparatus like that of SHA'AFI *et al.*[2]. In those experiments in which relaxations were definitely not observed we have placed a dash in

Table I. In all cases amplitudes of the $\tau_1$ curves were large compared to those of the longer $\tau_2$ relaxation. Thus the blanks in the $\tau_2$ column reflect irreproducibility of these longer curves. Each $\tau$ given is an average of at least three independent measurements.

The first 3 lines of data in Table I indicate that neither $\tau_1$ nor $\tau_2$ depends on the number of erythrocytes per unit volume. Clearly, at so high a dilution clumping of fresh erythrocytes is improbable.

TABLE I

LIGHT SCATTERING JOULE HEATING TEMPERATURE JUMP RELAXATIONS IN SUSPENSIONS OF HUMAN ERYTHROCYTES

All data obtained in water at 25°, pH 7.3, 0.9% NaCl and 90° scattering angle except as noted.

| *Experimental conditions* | $\tau_1$ (*msec*) | $\tau_2$(*msec*) |
|---|---|---|
| Erythrocyte dilution: 1/500 | 0.83 | 117 |
| 1/1000 | 0.83 | 123 |
| 1/1500 | 0.85 | 122 |
| 1/2000 | 0.80 | |
| Scattering angle 45°; 1/500 | 1.03 | 137 |
| Scattering angle 135°; 1/500 | 1.06 | 125 |
| Hemoglobin only (cells lysed and ghosts removed by centrifugation) | – | – |
| Ghosts only (cells lysed in distilled water and hemoglobin removed by washing in isotonic saline) | 0.88 | – |
| $^2H_2O$ solvent; erythrocyte dilution 1/500 (cells equilibrated 5 h in 0.9% saline 99.7% $^2H_2O$) | 1.21 | |
| NaCl: 0.45% (hypotonic), erythrocyte dilution 1/500 | 0.50 | |
| 0.90% (isotonic), erythrocyte dilution 1/500 | 0.97 | 115 |
| 1.80% (hypertonic), erythrocyte dilution 1/500 | 1.35 | |
| Sodium lauryl sulfate molarity ($\mu$M); erythrocyte dilution 1/500 | | |
| 0 (biconcave disc) | 0.91 | |
| 4 | 0.85 | |
| 20 (crenated disc) | 0.70 | |
| 40 | 0.58 | |
| 60 | 0.47 | |
| 80 | 0.43 | |
| 120 (crenated sphere) | 0.35 | |
| 160 (smooth sphere) | 0.25 | |
| 200 (ghost) | 0.16 | |
| 240 | 0.11 | |
| 160 $\mu$M 1,5-difluoro-2,4-dinitrobenzene; erythrocyte dilution 1/500 | 0.9 | |

Comparison of Lines 1, 5 and 6 indicates that there is no significant angular dependence of either relaxation. We cannot conclude from this that there are no major changes in erythrocyte shape since the time dependence (*i.e.* relaxations) would have to be the same at all angles.

The fact that we do not observe relaxations in lysed suspensions from which ghosts have been centrifuged (Line 7 of Table I) but which still contain hemoglobin indicates that hemoglobin alone is not responsible for our observed relaxations.

Since ghosts alone have the same $\tau_1$ as whole cells (see Line 8) but have no $\tau_2$, the second relaxation is apparently a property only of whole cells and not just the membrane.

When whole cells were rapidly immersed in 0.9 % NaCl in $^2H_2O$ we obtained a $\tau_1$ of approx. 0.9 msec. It was only when the cells had equilibrated long enough in $^2H_2O$ to have $^2H_2O$ inside as well as outside the membrane that we observed a lengthened $\tau_1$ of approx. 1.21 msec. This may indicate that we are observing a time constant for water transport through the membrane from the inside to the outside of the cell. A longer time constant for $^2H_2O$ transport through the cell membrane would be consistent with a higher coefficient of viscosity for $^2H_2O$ than for water (1.101 cP as opposed to 0.8937 cP at 25°)[9].

Aqueous isotonic NaCl, KCl, LiCl, and $Na_2SO_4$ suspensions of human erythrocytes at a dilution of 1/500 all gave the same relaxations as the first line of Table I to within experimental error. The same was true when we used the isosmolar buffer described by SHA'AFI *et al.*[2]. Thus neither $\tau_1$ nor $\tau_2$ can be attributed to selective transport of a particular ion through the membrane.

The data on several NaCl and sodium dodecyl sulfate concentrations can be considered together. Sodium dodecyl sulfate is well known to alter the shape of erythrocytes and in high enough concentration to lyse them. Our microscopic observations of cell shape noted in parentheses are consistent with those reported by DEUTICKE[10] and earlier by PONDER[11]. A decrease in NaCl or an increase in sodium dodecyl sulfate concentration can be crudely imagined to open up water transport passageways in the cell membrane as the cell distends preliminary to lysing. The smooth decrease in $\tau_1$ with increasing cell size observed in both cases is thus again consistent with the assumption that we are observing water passage through membrane pores with a consequent change in refractive index or cell shape that gives rise to a change in scattered light intensity.

Cross-linking of the erythrocyte membrane by 1,5-difluoro-2,4-dinitrobenzene reportedly[12] rigidified the membrane so that the cell will not lyse in distilled water even after many days. The fact that in 0.9 % NaCl and 16 $\mu$M difluoro compound $\tau_1$ remains the same suggests that a cell shape change is not responsible for the observed time dependence of scattered light intensity. When the medium is made hypo- or hypertonic the relaxation time shortens or lengthens, respectively, just as in the experiments (Lines 10–12 of Table I) wherein no difluoro compound was present. Evidently, then the difluoro compound is not effective in maintaining a particular pore size as saline concentration changes.

## DISCUSSION

The cellular membrane of a human erythrocyte is composed largely of proteins and lipids and is pierced by small aqueous channels through which neutral or negatively charged particles pass fairly readily[13]. In our experiments as temperature jumps in the surrounding saline solution because of Joule heating, an approx. 8° difference in temperature between the erythrocyte interior and the surrounding medium momentarily exists. The consequent difference in osmotic pressure inside the erythrocyte and out is given by $\Delta\pi = Rc\Delta T$, where $\pi$ = osmotic pressure; $R$ = the gas constant; $c$ = the electrolyte concentration; and $T$ = temperature. Since $\pi$ is momentarily greater outside the cell, we expect a tendency for water molecules inside the cell to pass through the membrane to the exterior. The consequent changes in refractive indices of the saline solutions inside and outside the erythrocyte would cause a concurrent change in scattered light intensities regardless of whether the erythrocyte volume changes or not.

This picture raises a key question: Does $\Delta T$ persist longer than the half-life for water passage through the biological membrane. If so, the 0.9-msec relaxation time characterizes water passage through the membrane. If not, we have simply observed a relaxation characteristic of temperature reequilibration between the interior and exterior of the cell and water passage is characterized by a still shorter relaxation time.

Crude calculations based on a cell diameter of approx. 7 $\mu$ and a membrane thickness of approx. 100 Å suggest that conductive heat transport to the erythrocyte interior may have a time constant as short as 1 msec. We have found no experimental evidence on the rate of such a thermal equilibration process. If the hemoglobin inside the erythrocyte undergoes a slight color change with temperature, it may be possible to determine the time constant for interior heating spectrophotometrically. For such a temperature jump determination of the time constant for temperature reequilibration it would be essential that the erythrocytes not burst. Unfortunately, high electric fields like those used here (approx. 30kV/cm) lyse erythrocytes. Thus we presently do not know how to obtain this one vital piece of information.

On the subject of erythrocyte lysis in intense electric fields, it has been noted that ten 4-kV/cm pulses 20 $\mu$sec in duration and 1 sec apart achieve 100 % lysis of bovine erythrocytes[14]. We found that human and frog erythrocytes are much more susceptible to electric field lysis than are rat erythrocytes. In the case of human erythrocytes, our $\tau_2$ of approx. 120 msec relaxation disappears after six 30 kV/cm pulses of approx. 10 $\mu$sec duration but the 0.9-msec relaxation does not. This result supports our earlier conclusion that the longer relaxation $\tau_2$ is a property of unruptured erythrocytes only.

The principal conclusion of this work is, that the Joule heating temperature jump relaxation method of EIGEN AND DE MAEYER[4] with spectrophotometric detection of changing scattered light intensities has interesting possibilities for investigating the kinetic properties of cell membranes. We have incidentally measured similar relaxation with bacteria and algae. We also tentatively conclude that the time constant for transmission of water through the human erythrocyte membrane is 0.9 msec at 25° in isotonic saline solution. However, this may only be a lower limit if thermal reequilibration of the erythrocyte interior with the surrounding saline solution has a time constant as short as this.

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