and M. Cohn for myeloma tumors and to D. Knecht for DNA from a mouse β-globin clone which was produced by H. Faber, L. Beach, F. Blattner, L. Furlong, J. Ross, and O. Smithies (unpublished experiments).

References

Bernard, O., Hozumi, N., & Tonegawa, S. (1978) *Cell 15*, 1133.
Brack, C., & Tonegawa, S. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 5652.
Brack, C., Hirama, M., Lenhard-Schuller, R. & Tonegawa, S. (1978) *Cell 15*, 1.
Dreyer, W. J., & Bennett, J. C. (1965) *Proc. Natl. Acad. Sci. U.S.A. 54*, 864.
Gilmore-Herbert, M., Hercules, K., Komaromy, M., & Wall, R. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 6044.
Gross-Bellard, M., Oudet, P., & Chambon, P. (1973) *Eur. J. Biochem. 36*, 32.
Jeffreys, A. J., & Flavell, R. A. (1977a) *Cell 12*, 429.
Jeffreys, A. J., & Flavell, R. A. (1977b) *Cell 12*, 1097.
Lenhard-Schuller, R., Hohn, B., Brack, C., Hirama, M., & Tonegawa, S. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 4709.
Maniatis, T., Jeffreys, A., & van deSande, H. (1975) *Biochemistry 14*, 3787.
Pernis, B., Chiappino, G., Kelus, A. S. & Gell, P. G. H. (1965) *J. Exp. Med. 122*, 853.
Rigby, P. W. J., Dieckmann, M., Rhodes, C., & Berg, P. (1977) *J. Mol. Biol. 113*, 237.
Seidman, J. G., & Leder, P. (1978) *Nature (London) 276*, 790.
Seidman, J. G., Leder, A., Edgell, M. H., Polsky, F., Tilghman, S. M., Tiemeier, D. C., & Leder, P. (1978a) *Proc. Natl. Acad. Sci. U.S.A. 75*, 3881.
Seidman, J. G., Leder, A., Nau, M., Norman, B., & Leder, P. (1978b) *Science 202*, 11.
Southern, E. M. (1975) *J. Mol. Biol. 98*, 503.
Storb, U. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 2905.
Storb, U., Hager, L., Wilson, R., & Putnam, D. (1977) *Biochemistry 16*, 5432.
Tonegawa, S., Brack, C., Hozumi, N., Matthyssens, G., & Schuller, R. (1977) *Immunol. Rev. 36*, 73.
Valbuena, O., Marcu, K. B., Weigert, M., & Perry, R. P. (1978) *Nature (London) 276*, 780.
Wilson, R., Walfield, A. M., Murakami, A., & Storb, U. (1978) *Biochemistry 17*, 3096.

Internal Microviscosity of Red Blood Cells and Hemoglobin-Free Resealed Ghosts: A Spin-Label Study†

Philip D. Morse II,* Donna M. Lusczakoski,‡ and David A. Simpson§

ABSTRACT: Although important for the understanding of intracellular events, the internal viscosity of red blood cells is not known. We present electron spin resonance data which show the effect of cell size, extracellular pH, and temperature on the motion of a small spin-label, 4-amino-2,2,6,6-tetramethylpiperidinyl-*N*-oxy (Tempamine), both in the presence and absence of hemoglobin. Tempamine motion in the interior of intact red blood cells is hindered by a factor of 5.47 ± 2.04 at pH 7.5, 25 °C, and 320 mosm, while in resealed ghosts Tempamine motion is hindered by a factor of 2.34 ± 0.42. Both values decrease with increasing cell size, increase with increasing pH (to pH 9), and decrease with increasing temperature. Further data exclude the possibility that binding of Tempamine to red cell membranes or hemoglobin could account for our results. Tempamine is apparently randomly distributed throughout the red cell interior both in the absence and presence of hemoglobin. We suggest that the aqueous interior of both intact and resealed red blood cells and ghosts hinders the motion of Tempamine. This further suggests that the microviscosity inside the red cell and resealed ghosts is greater than that of bulk water.

The internal viscosity of cells has been shown to be greater than that of bulk water by a number of methods, such as nuclear and proton magnetic resonance [see Cooke & Kuntz (1974) and Hazlewood (1973) for reviews] and more recently by electron spin resonance (Keith & Snipes, 1974; Morse et al., 1975; Henry et al., 1976; Haak et al., 1976; Keith et al., 1977a; Morse, 1977; Berg et al.,1979). The results obtained by these techniques show that water diffusion and spin-label rotation are hindered inside cells and organelles by factors of 2–40 compared to bulk water (depending on the method). The source of this increased intracellular viscosity is unknown.

Recent evidence suggests that the cell membrane may contribute in some way to the intracellular viscosity. Morse et al. (1975) found that intravesicular viscosity of lobster sarcoplasmic reticular vesicles was ~20–40 times greater than that of bulk water. This was not caused by proteins in the internal aqueous space, and they concluded that the inner surface of the membrane was influencing the order of the internal aqueous environment. Further studies by Berg et al. (1979) on spinach thylakoids suggested that the membrane-associated proteins influence the apparent viscosity of intracellular water.

A previous study by Dintenfass (1968) suggests that maintenance of the internal viscosity of the red blood cell at some nominal value is required for proper circulatory function. The values for internal viscosity obtained by Dintenfass are,

† From the Department of Biological Sciences, Wayne State University, Detroit, Michigan 48202. *Received February 9, 1979; revised manuscript received April 26, 1979.* This research was supported in part by contract N00014-76-C-1167 from the Office of Naval Research.

‡ Present address: Department of Biological Sciences, University of Denver, Denver, CO 80208.

§ Present address: Kirksville School of Osteopathic Medicine, Kirksville, MO 63501.

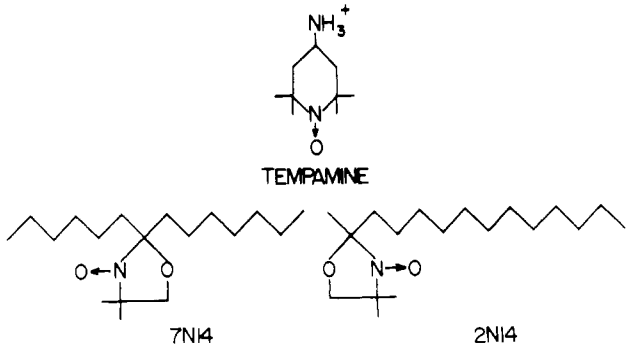
0006-2960/79/0418-5021$01.00/0

however, 1–3 orders of magnitude larger than the values quoted in the first eight references for other cell types (including red blood cells). In addition, Dintenfass used viscometric methods which cannot distinguish between internal viscosity and membrane viscosity.

We seek to answer the question left unanswered by Dintenfass concerning the relationships between the red cell membrane and internal viscosity. We also wish to answer questions dealing with the origin of intracellular viscosity in the red blood cells as observed by electron spin resonance, specifically, whether the hindered rotation of spin-labels inside the red blood cell (Morse, 1977) is due to intracellular hemoglobin, the membrane, or a combination of both. We make use of the fact that red blood cell ghosts can be prepared which are entirely free of homoglobin but are still essentially impermeable to ions, specifically potassium ferricyanide (Morse et al., 1978). In the course of this study, intracellular spin-label motion was examined as a function of pH, temperature, and osmolarity since these are normal biological variables to which red blood cells are exposed. Two membrane spin-labels were used to monitor changes in the membrane polar and hydrocarbon regions under these same variables. The effect of hemoglobin on intracellular viscosity is analyzed by comparing the results obtained from spin-labeled intact red blood cells with those obtained from spin-labeled hemoglobin-free resealed ghosts. The data presented and analyzed here show that both the red cell membrane by itself and with internal hemoglobin hinders the rotation of spin-labels in the intracellular aqueous region. This hindrance is not due to the binding of the spin-label to either hemoglobin or the red cell membrane.

Materials and Methods

All chemicals used in this study were AR grade. The spin-label Tempamine was synthesized by the method of



Rozantsev (1970) and stored as a 100 mM solution in water at pH 7.5. The membrane spin labels 2N14 and 7N14 were synthesized according to the methods outlined by Williams et al. (1971) and purified by the methods of Morse, Kavieff, and Lusczakoski (unpublished experiments). Red blood cells were obtained from the Detroit Red Cross and were used well before their expiration date. Preparation of the red cells, the hemoglobin-free resealed ghosts, and purified hemoglobin has been previously described (Morse et al., 1978). Total protein and hemoglobin concentrations were measured by the methods of Lowry et al. (1951) and Drabkin & Austin (1932), respectively. Spectrophotometric assays of protein and hemoglobin solutions were made on a GCA/McPherson EU-721 spectrophotometer.

Electron Spin Resonance. ESR spectra were recorded with a Varian E-109 E spectrometer at the following settings: modulation amplitude at 0.5 G; power 5 mW; scan times 2–4 min; and time constants of 0.128–0.5 s. The spectrometer was equipped with an E-238 TM cavity and an E-257 temperature controller.

The ESR experiments described in this paper were of two types. The first type examined the motion of a water-soluble spin-label, Tempamine, in the intracellular compartment of the red blood cell or resealed ghost. Potassium ferricyanide (Mallinckrodt) was used to broaden the Tempamine signal not associated with the internal space. This method is described in full detail by Morse (1977). In the second type of experiment, the membrane spin-labels 2N14 and 7N14 were used to probe the polar and hydrocarbon membrane regions, respectively. The location and distribution of these spin-labels in the membrane plane have been previously established (Morse et al., 1975).

The ESR samples containing the water-soluble spin-label Tempamine were prepared as follows. One microliter of 100 mM Tempamine in water was placed in the bottom of a 10 × 75 mm test tube and 99 μL of the red cell or resealed ghost suspension containing 80 mM $K_3Fe(CN)_6$ was added. All samples were then mixed and drawn up into a 1-mm i.d. glass capillary, flame-sealed, and placed inside a 3-mm i.d. quartz tube before insertion in the ESR cavity. No attempt was made to exclude oxygen from the system. HbO_2 does not affect the line widths of small ESR spin-labels (Beaudoin & Mizukami, 1978). Temperatures of the samples in the cavity were controlled by a nitrogen gas flow system and monitored with a copper–constantan thermocouple placed against the sample in the quartz tube. Temperature fluctuations at a single point were less than 0.05 °C, but there was a large temperature gradient (~2 °C) along the sample. Care was used to ensure that the thermocouple was measuring the temperature at the active region of the sample, that is, where the ESR signal was being measured. This distance was ~1 cm along the length of the sample, and the variation in temperature over this distance was on the average of ±0.5 °C.

Rotational correlation times (τ_c) were calculated by using the approximations described by Keith et al. (1970) which yield the equation

$$\tau_c = kW_0[(h_0/h_{-1})^{1/2} - 1]$$

where k is a constant which depends on the values of the g and hyperfine tensors for the spin-label and the microwave frequency. In the absence of crystal parameters for the hyperfine and g values, we used $k = 6.5 \times 10^{-10}$ (Morse et al., 1975). The other parameters, W_0, h_0, and h_{-1}, are the first derivative peak-to-peak mid-field line width, mid-field line height, and high-field line height, respectively. The integrated intensity was calculated from the equation $A_0 = W_0^2 h_0$/instrument gain, where A_0 is the area under the first derivative mid-field line.

The internal microviscosity reported by Tempamine inside the red cells or resealed ghosts was obtained by comparison of the rotational correlation time of Tempamine in water to that of Tempamine in the samples. Rotational correlation time is also used to measure the motion of 2N14 and 7N14, but the motion of these spin-labels in the membrane is not nearly as isotropic as the motion of Tempamine (see Results). Therefore, the measurements of τ_c for each membrane spin-label have validity only as indicators of relative motion.

Use of the Descriptive Term "Internal Microviscosity". The problems associated with the term internal microviscosity have been discussed previously (Morse et al., 1975). In general, viscosity measurements using spin-labels require that the solvent be uniform and isotropic and that no interactions occur between the solvent and the spin-label. In certain cases, we can only assume that these requirements are satisfied, and

because of this we use a comparative analysis of viscosity and define the term $\eta\mu$, where $\eta\mu$ is obtained from the ratio of the rotational correlation time of Tempamine in the cell to the rotational correlation time of Tempamine in water at 25 °C (4×10^{-11} s), as $\eta\mu = \tau_c(\text{cell})/\tau_c(\text{water})$. This is a relative measurement and does not give absolute values of the viscosity inside the cell. In addition, any given Tempamine molecule can only sample a small region inside the cell. Although Tempamine rotation is related to bulk viscosity in a linear manner (Morse et al., 1975; Berg et al., 1979), Tempamine only provides information about a microenvironment and hence only yields data about microviscosity. This does not imply that macromolecules would be subject to the same motional constraints as Tempamine. However, the relative microviscosities obtained from this technique will reliably indicate increases or decreases in $\eta\mu$ under variable experimental conditions.

Establishment of Osmotic Strength and pH in the Samples. Osmotic strength was varied by washing the red cells or resealed ghosts in a 30-fold excess of 5 mM sodium phosphate buffer, pH 7.5, containing the appropriate concentrations of potassium ferricyanide and sodium chloride. They were then centrifuged at 7000*g* for red cells and 25000*g* for resealed ghosts for 10 min at 4 °C. For osmotic strengths below 320 mosm, it was necessary to use potassium ferricyanide exclusively in decreasing amounts. However, sufficient quantities of cells were used (hematocrit ≥80%) so that the decreased ferricyanide concentrations did not alter the degree to which the extracellular Tempamine contributed to the final spectra. Extracellular ferricyanide-broadened Tempamine contributed less than ~1% to the total signal height of Tempamine in intact red blood cells and less than ~10% for the resealed ghosts. Because the ferricyanide-broadened Tempamine signal was extremely broad, it had no obvious measurable influence on the calculations of τ_c. For osmotic strengths above 320 mosm, 80 mM potassium ferricyanide and the appropriate concentrations of sodium chloride were used. The pH and temperature were carefully controlled during the osmolarity studies. The pH was established by washing the red cells or resealed ghosts 3 times in a 30-fold excess of 5 mM sodium phosphate buffer of varying pH to which potassium ferricyanide was added in sufficient quantities to maintain 320 mosm. The pH of the Tempamine solution was also adjusted accordingly. Temperature was maintained at 25 °C unless stated otherwise. An Advance osmometer and a Radiometer pHM 26 pH meter were used to measure osmolarity and pH, respectively.

Results

Spectra of Tempamine in Intact Red Blood Cells and Resealed Ghosts. Figure 1 shows the spectrum of 1 mM Tempamine in (A) PBS at pH 8, (B) 80 mM ferricyanide, (C) 80 mM ferricyanide with intact red blood cells, and (D) 80 mM ferricyanide with resealed ghosts and (E) a computer subtraction of spectrum D minus spectrum B. The Tempamine signal from intact red blood cells did not change with time (over 1 h), but that of the resealed ghosts did. If ferricyanide was added directly to the ghosts instead of using our washing procedure, the Tempamine signal height would decrease by up to a factor of 2 over a period of 5–10 min (Figure 2A). However, repeated scans of the resealed ghosts (Figure 2B) indicated that there was no change in line width.

It was possible to reconstruct the line heights of the original sample by measuring the decrease of each line with time after the addition of ferricyanide. This was accomplished by preparing a sample and scanning with the magnet field sweep

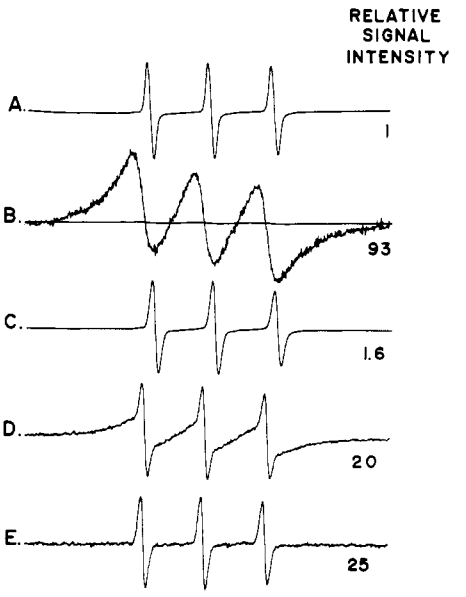


FIGURE 1: ESR spectra of Tempamine. All spectra were taken at a microwave power of 5 mW and a modulation amplitude of 0.5 G. The field center was 3197.4 G, the field sweep was 100 G, and the microwave frequency was ~8.97 GHz. Spectra are normalized to the same peak-to-peak height of the mid-field line. The relative instrument gain necessary for this normalization is listed to the right of each spectrum. (A) Spectrum of Tempamine in 0.15 M NaCl and 5 mM phosphate buffer, pH 8. The peak-to-peak height of the mid-field line is used as the reference point for all other spectra. (B) Spectrum of 1 mM Tempamine in 80 mM $K_3Fe(CN)_6$ and 5 mM phosphate buffer, pH 8, at the same receiver gain setting as (A) (flat line) and normalized to (A). Note that the intensity of this signal is very much reduced relative to (A) by line broadening. (C) Same as (B) except for the addition of intact red blood cells. Relative gain is arbitrary in this case since virtually all of the Tempamine signal arises from inside the cell and is dependent on the hematocrit. In this case, the hematocrit is ~63%. τ_c is 2.0×10^{-10} s. (D) Same as (B) except for the addition of resealed red blood cell ghosts at ~2 mg of membrane protein per mL of sample. The ghosts have been exposed to ferricyanide for ~10 minutes (see text). τ_c is 8.3×10^{-11} s. (E) Spectrum of red cell ghosts with the ferricyanide-broadened Tempamine signal subtracted by digital methods (spectrum D minus spectrum B). Spectrum B was first adjusted in amplitude to correspond to the gain setting of spectrum D. τ_c is 8.1×10^{-11} s.

at zero and the magnet field setting centered on the peak of the mid-field line. The resultant curve was then linearized by plotting the inverse of the signal intensity vs. the inverse of time, and the spectral line height was calculated at time zero. Because there was no line width change, it was possible to calculate a τ_c from the sample at time zero. Typical values were 8×10^{-10} s. Thus, τ_c did not change with time in the resealed ghosts.

Integration of the spectra showed that the total observable Tempamine concentrations did not change with time. Figure 2C shows the first integral of Tempamine in resealed ghosts immediately after addition of ferricyanide (time after addition of ferricyanide spans 60–140 s over the width of the spectrum) and after 10 min. Spectrometer settings were the same in both cases. Double integration reveals that the identical amount of Tempamine is present in both cases. The spectra of Figure 2C consist of three sharp peaks on top of three broad peaks which are on a large "hill". The three broad peaks and the hill represent extracellular Tempamine broadened by ferricyanide, and the sharp peaks represent unbroadened intracellular Tempamine (Figure 2D).

Because all the Tempamine can be accounted for, the loss of the Tempamine signal from the resealed ghost sample is not due to reduction of the spin-label. These results can be explained by assuming that there is a fraction of the ghosts

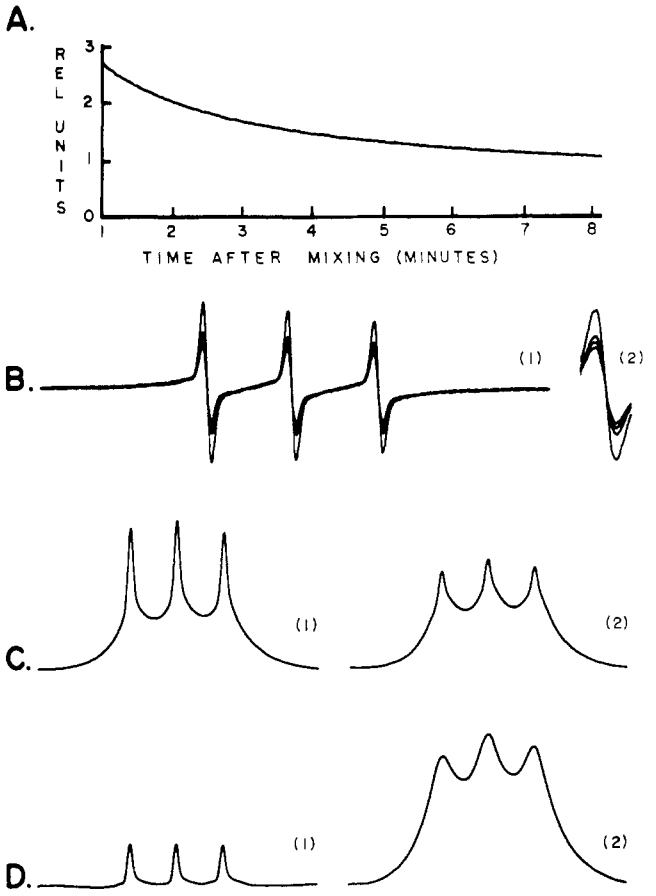


FIGURE 2: Loss of Tempamine signal with time in resealed ghosts. (A) Loss of signal intensity of the mid-field line with time. The field scan was set to zero and the field centered on the peak of the mid-field line (3197 G at ~8.97 GHz). When replotted as 1/intensity vs. 1/time, a straight line explained greater than 99% of the variance. Using the parameters obtained by this procedure, it was possible to calculate the line height at the moment of ferricyanide addition. (B) Successive scans of Tempamine in the presence of ghosts and 80 mM ferricyanide. (Part 1) 100-G sweep and (part 2) 10-G sweep of mid-field line. Note that there is no change in line width with time. From the data in (A) and (B), τ_c at the instant of ferricyanide addition can be calculated. A typical value was 8×10^{-11} s. (C) First-integral plot of (B). (Part 1) From 1 to 1.5 min; (part 2) from 10 to 10.5 min. Double integration and subtraction show that the total intensity under the broad peaks and hill is 90% in part 1 and 97% in part 2. Total double-integral values were equal. This shows a shifting of part of the Tempamine signal from the unbroadened to the broadened state. (D) First-integral plots of intracellular Tempamine alone (part 1) and 1 mM Tempamine in 80 mM ferricyanide (part 2). These plots show that the broad peaks and hill in (C) arise from ferricyanide-broadened Tempamine and the sharp peaks from unbroadened intracellular Tempamine.

which does not reseal to ferricyanide even after overnight incubation at 0 °C. Thus, signal loss is due to leakage of ferricyanide into these (as yet) unsealed cells. Because the sharp signal from which τ_c was measured changed only quantitatively (signal height decreased) and not qualitatively (no change in τ_c or line width), this rules out the possibility that all resealed ghosts were partially leaky to ferricyanide. Samples in which the loss of signal height was greater than a factor of 2 were not used for subsequent experiments. Samples which passed this test were washed in 80 mM ferricyanide and centrifuged for 10 min for subsequent experiments so that this initial signal loss is entirely gone by the time we make our measurements (see Materials and Methods).

The calculated internal microviscosities ($\eta\mu$) of intact red blood cells and hemoglobin-free resealed ghosts from 15 to 20 samples at 320 mosm, pH 7.5, and 25 °C were 5.47 ± 2.04 and 2.34 ± 0.42, respectively. These values of microviscosity are significantly greater than the microviscosity of bulk water but are much less than the values determined by Dintenfass (1968). They result from the restricted motion of Tempamine inside the red cells and resealed ghosts. This restricted motion may be a result of either the binding of the spin-label to some intracellular component such as hemoglobin or a membrane protein or a generally high intracellular microviscosity. The following experiments were designed to distinguish between these possibilities.

Binding of Tempamine. Tempamine which is bound to some intracellular or membrane component may alter the distribution of Tempamine such that the spin-label would accumulate inside the cells. This possibility was studied by incubating intact red blood cells, unsealed ghost membranes, and resealed ghosts in 1 mM Tempamine for 1 h followed by rapid centrifugation and immediate analysis of the supernatant by ESR. Whole cells were used to determine the binding of Tempamine to the hemoglobin as it is arranged inside the cell while leaky ghosts were used to study Tempamine binding to the membrane alone. The resealed membrane preparations were used to eliminate the possibility of nonspecific accumulation of Tempamine by ion gradients across the cell membrane in the intact or resealed cells.

The concentration of Tempamine in the suspension of cells was taken as unit value, and the concentration of Tempamine in the supernatant was compared to this. The pellets could not be examined directly because they were too viscous to draw into the sample capillary tube.

If Tempamine was bound to either intracellular hemoglobin or the membrane, then there would be less Tempamine in the supernatant than in an equivalent volume of the total cell suspension. The results show that, within error, there is no difference in the percent concentration of Tempamine in the supernatants relative to the suspension supernatants of the red blood cells (98.7% ± 6.2%), the unsealed ghosts (98.1 ± 2.0%), or the resealed ghosts (99.3 ± 0.7%).

The above experiment demonstrates equal distribution of Tempamine between the cells and the external solution, but it is still possible that Tempamine is asymmetrically distributed inside the red cells or resealed ghosts. This would again be caused by spin-label which is bound either to hemoglobin or to the membrane. Unequal distribution would give rise to regions of increased spin-label concentration such that collisions between spin-labels would be more frequent and broadening of the spectral lines would result (Morse et al., 1975; Kivelson, 1960; Dix et al., 1978). This would increase the line width of the 1 mM Tempamine signal beyond that expected from motional considerations alone.

Tempamine line widths were measured for red cells, resealed ghosts, and 3 and 12% hemoglobin to test this. The line widths expected from Tempamine rotation alone are measured from spectra of 1 mM Tempamine in glycerol–water mixtures of 5 cP for the red cells and 2 cP for the resealed ghosts. Line width data are normalized in this manner because line width is dependent on spin-label motion, which is in turn dependent upon viscosity (Morse et al., 1975; Kivelson, 1960; Dix et al., 1978). The mid-field line was chosen for these measurements since it is least affected by variations in spin-label motion (Keith et al., 1970; Kivelson, 1960). The average mid-field line width of 1 mM Tempamine inside the red blood cells was 1.57 ± 0.07 G and may be compared to 1.60 G for 1 mM Tempamine in a glycerol–water mixture with a bulk viscosity of 5 cP. The mid-field line width obtained from resealed ghosts was 1.63 ± 0.12 G and may be compared to 1.54 G for a

Table I: Microviscosity as a Function of the Tempamine/Hemoglobin Ratio[a]

[hemoglobin]/[Tempamine]	$\eta\mu$
500	3.0 ± 0.2
167	2.7 ± 0.2
50	3.0 ± 0.1
16.7	2.9 ± 0.1
5	2.8 ± 0.1
1.67	3.0 ± 0.2

[a] Tempamine concentrations were varied between 0.01 and 3.0 mM in 27% hemoglobin in 5 mM phosphate buffer.

glycerol–water mixture with a bulk viscosity of 2 cP. Both 3 and 12% hemoglobin gave line widths of 1.53 ± 0.01 G which can be compared to the line widths obtained in glycerol–water at 1.2 and 3.1 cP (1.51 and 1.53 G), respectively. Because there is no statistical difference between line widths of Tempamine in the resealed or normal cells compared with glycerol–water, these results suggest that Tempamine is randomly distributed inside the red cells and resealed ghosts.

Strong binding between Tempamine and hemoglobin may be further examined by an analysis of the spectral line shapes. The spectra did not show any immobilized component; however, binding between Tempamine and hemoglobin may still occur as a rapid equilibrium. Evidence of equilibrium binding may be detected by changes in the rotational correlation time of Tempamine in 27% hemoglobin as a function of Tempamine concentration. A hemoglobin concentration of 27% was chosen to mimic the concentration of hemoglobin in the red blood cell. By assumption of rapid equilibrium between free and bound Tempamine such as

$$\text{Tempamine} + \text{hemoglobin} \rightleftharpoons \text{Tempamine–hemoglobin}$$

then lower ratios of Tempamine/hemoglobin should shift the equilibrium away from the formation of hemoglobin–Tempamine complexes. The unbound Tempamine would then give rise to a decrease in the apparent rotational correlation time of Tempamine. Similarly, higher ratios should increase the apparent rotational correlation times of Tempamine.

The data presented in Table I correspond to hemoglobin/Tempamine ratios ranging from about 500:1 to 5:3. There is no change in $\eta\mu$ over the range of Tempamine concentrations studied. This suggests that equilibrium binding between Tempamine and hemoglobin molecules lies far to the left under the conditions of this study, and hence no binding is observed.

We conclude from the above studies that the motion of Tempamine inside red blood cells and ghosts is an actual reflection of the intracellular viscosity and is not caused by binding of the spin-labels either to the membrane or to hemoglobin. This allows us to use Tempamine to probe the microviscosity of the red blood cells and resealed ghosts under varying conditions of osmolarity, pH, and temperature.

Osmolarity. The microviscosity of the aqueous interior of the intact red blood cell as monitored by Tempamine is shown to increase with increasing osmolarity in Figure 3A. When viewed by light microscopy, the intact cells showed the expected shape changes as a function of osmotic strength. At 600 mosm the cells were predominantly crenated, while at 170 mosm the cells were predominantly spheroid. Tempamine reports an increasingly viscous environment as the cells shrink. A similar result was found for Tempamine motion in resealed ghosts. However, Tempamine tumbled about 2.5 times more rapidly in the ghost interior compared to the intact red blood cell interior.

The effect of osmolarity on the membrane spin-labels is shown in Figure 3B. 2N14 shows no change in rotational

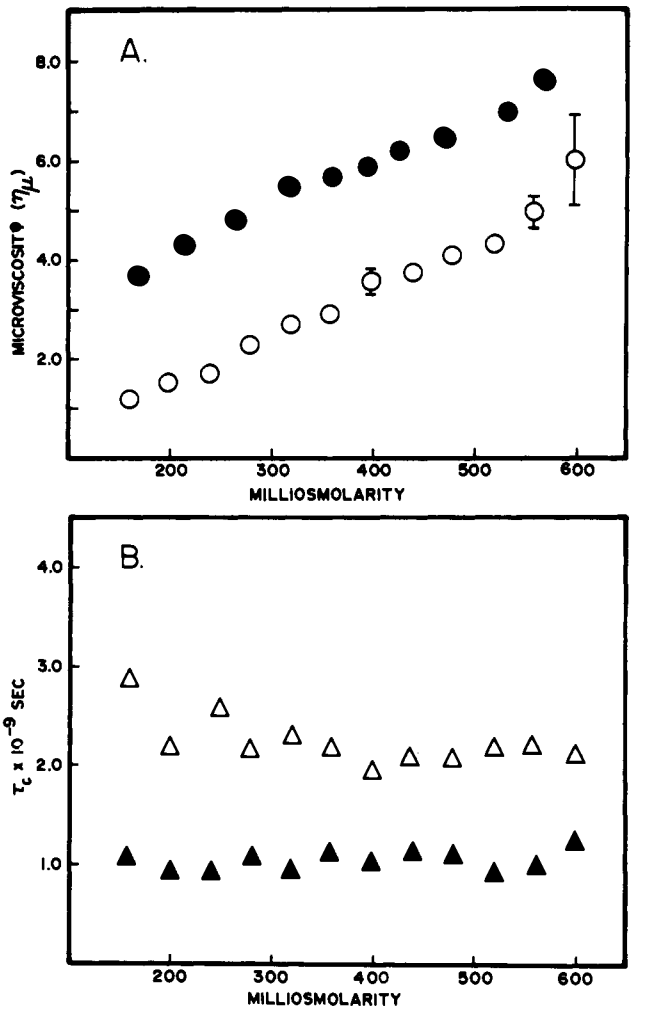


FIGURE 3: (A) Internal microviscosity of red blood cells and hemoglobin-free resealed ghosts as a function of osmolarity. All data on internal viscosity are plotted with respect to the internal viscosity at 320 mosm, pH 8.0, and 25 °C. (●) Red blood cells; (○) resealed ghosts. (B) Rotational correlation times of the spin-labels 2N14 and 7N14 in the red cell membrane as a function of osmolarity. (▲) 2N14; (△) 7N14.

motion within experimental error, while 7N14 shows a slight decrease in correlation time (increase in motion) from about 170 to 400 mosm. Since the changes in internal microviscosity do not correspond to the changes in the motion of the membrane spin-labels, it seems unlikely that the membrane regions monitored by 2N14 and 7N14 are involved with the observed changes in microviscosity reported by Tempamine.

pH. The influence of pH on microviscosity is shown in Figure 4A. There is a considerable rise in microviscosity between pH 5.5 and 8.0, but above pH 8.0 no further changes occur. Data obtained at pH 11 (not shown) also yielded a value of ~6 for the microviscosity. The microviscosity of the resealed ghosts shows very little change as a function of pH. There is a slight rise from pH 5.0 to 8.0, but this is not nearly as large as the increase observed for the intact cells. A small decrease in microviscosity is noted between pH 8.0 and 9.0. Both the intact cells and resealed ghosts have a maximum microviscosity at about pH 8.0.

The membrane spin-labels showed no change in motion between pH 5.0 and 9.0 (Figure 4B). Changes in the charges of the phospholipids do not occur over this pH range, although conformational changes are occurring in the membrane proteins (Hanahan, 1973). These changes do not seem to affect those membrane regions sampled by 2N14 and 7N14.

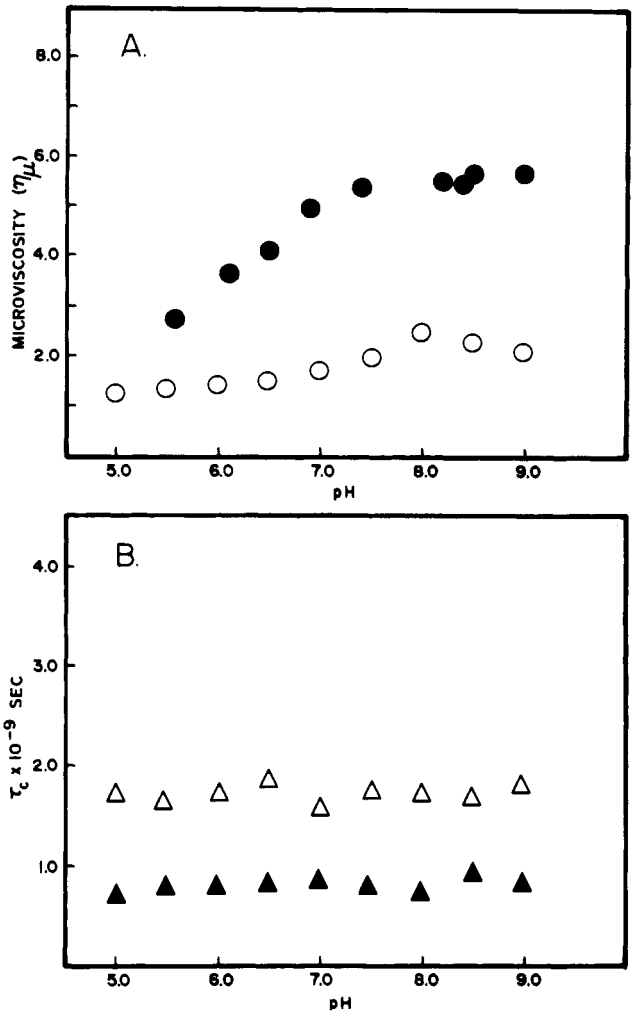


FIGURE 4: (A) Internal microviscosity of red blood cells and hemoglobin-free resealed ghosts as a function of pH. (●) Red blood cells; (○) resealed ghosts. (B) Rotational correlation times of the spin-labels 2N14 and 7N14 in the red cell membrane as a function of pH. (▲) 2N14; (△) 7N14.

Temperature. Temperature affects the bulk viscosity of water, and consequently there is an effect on the rotational correlation time of Tempamine in water. Figure 5 shows that $\eta\mu$ decreases smoothly as temperature increases. This indicates that changes in the viscosity of water which occur as a function of temperature are accurately monitored by Tempamine. However, since the viscosity of water changes as a function of temperature, the measurements of internal microviscosity as a function of temperature were analyzed in a slightly different manner than the measurements of the pH and osmolarity dependency. Instead of calculating all the microviscosities relative to the correlation time of Tempamine in water at 25 °C, the microviscosities were calculated from the correlation times of Tempamine at the corresponding temperature. If Tempamine motion in the cells was influenced by temperature in the same manner as in bulk water, the data should plot as a straight line. This was the result obtained for the red blood cells over the temperature range of 5–40 °C, as shown in Figure 6A. The results for the resealed ghosts are essentially the same, except that there is an increase in microviscosity between 30 and 40 °C. However, these data are probably less reliable than the red blood cell data since the resealed ghosts become leaky to ferricyanide at higher temperatures.

The motion of the membrane spin-labels as a function of temperature is shown in Figure 6B. In both cases the spin-

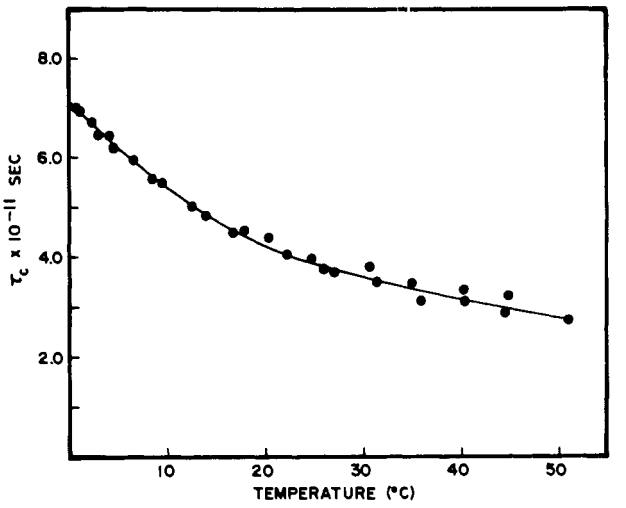


FIGURE 5: Rotational motion of Tempamine in bulk water at various temperatures. When τ_c is plotted against bulk viscosity, the line connecting the points is straight (not shown). These values are used for obtaining reference points for comparing the motion of Tempamine in the red cells and resealed ghosts at different temperatures.

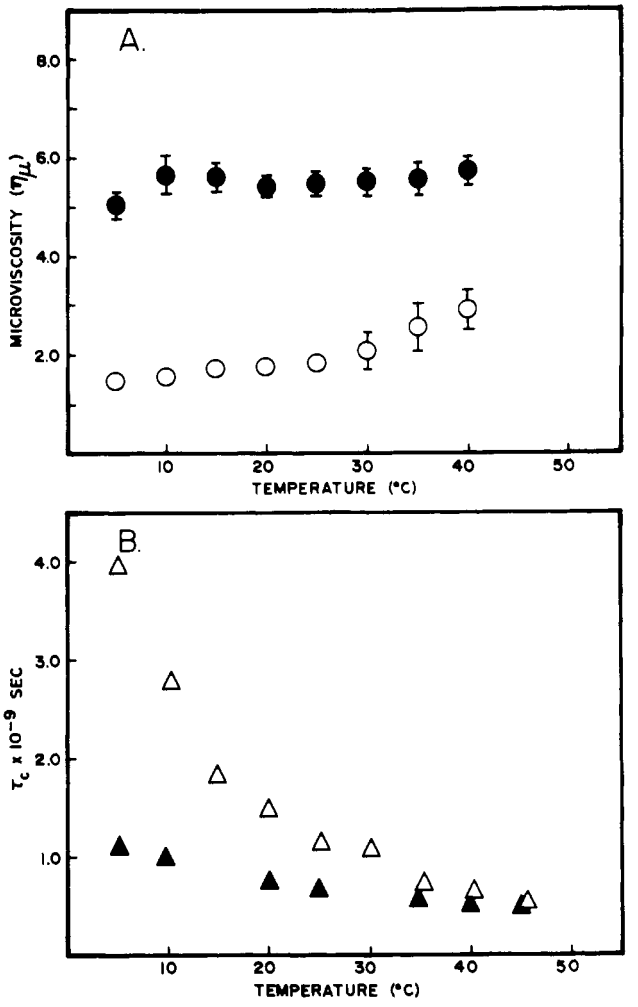


FIGURE 6: (A) Internal microviscosity of red blood cells and hemoglobin-free resealed ghosts as a function of temperature. Data points were calculated with respect to the rotational correlation time of Tempamine in water at similar temperatures. (●) Red blood cells; (○) resealed ghosts. (B) Rotational correlation times of the spin-labels 2N14 and 7N14 in the red cell membrane as a function of temperature. (▲) 2N14; (△) 7N14.

labels are responding to changes in membrane fluidity brought about by changes in temperature. However, the dramatic

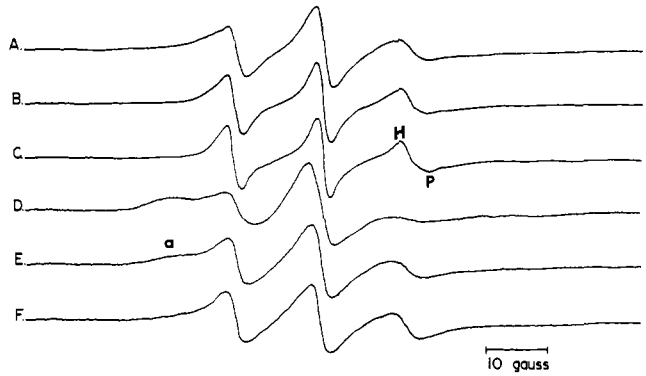


FIGURE 7: ESR spectra of 2N14 and 7N14 at 5, 20, and 37 °C. Spectra A and D are of 2N14 and 7N14 at 5 °C, spectra B and E are of 2N14 and 7N14 at 20 °C, and spectra C and F are of 2N14 and 7N14 at 37 °C. Note in spectrum D that 7N14 gives the indication of spinning anisotropically in the plane of the membrane as shown by the characteristic "hump" (a) which indicates that a spin-label is tumbling with some degree of anisotropy in the z axis of the molecule. This disappears at 37 °C. Spectrum C shows the slight partitioning which is characteristic of 2N14 in membrane samples. This indicates that the spin-label is distributed between the aqueous phase and the membrane phase of the sample. Vestiges of this effect are observed at 20 °C and even at 5 °C. H arises from 2N14 in the membrane while P arises from 2N14 in the aqueous regions of the sample.

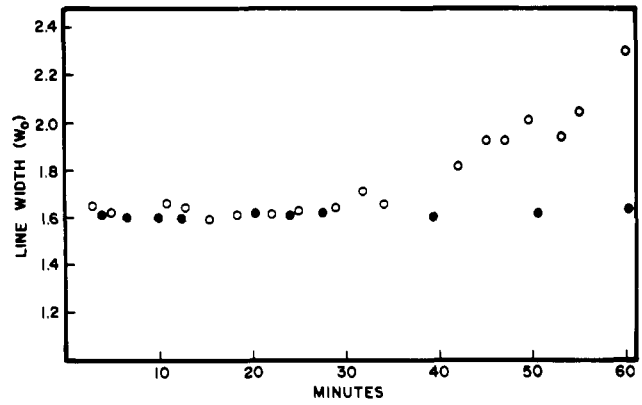


FIGURE 8: Changes in line width of Tempamine inside red blood cells and resealed ghosts. Line broadening is an indication of ferricyanide leakage. Line widths of Tempamine in intact red blood cells did not change over a period of 80 min, suggesting that ferricyanide does not penetrate intact red blood cells over this period. However, line width changes were apparent in the resealed ghosts, but only after 0.5 h of incubation with 80 mM ferricyanide. Thus, leakage of ferricyanide does not affect our results during the time frame of our experiments (15 min at the most after mixing of the cells or ghosts with ferricyanide).

decrease in τ_c for 7N14 between about 5 and 20 °C is related to the relatively anisotropic motion of 7N14 in the hydrocarbon region of the red cell membrane at low temperature (see below). The motional changes of 2N14 are not as dependent on temperature, but both labels do indicate (by a decrease in τ_c) that the membrane fluidity of the red blood cell is decreasing as the temperature increases.

Examples of spectra of 2N14 and 7N14 in the red cell membrane at 5, 20, and 37 °C are shown in Figure 7. The letter "a" indicates a spectral intensity of 7N14 which probably arises from motional anisotropy (Seelig, 1976) at low temperatures. This feature becomes much smaller at 20 °C and is undetectable at 37 °C. The letters "H" (hydrocarbon) and "P" (polar) in the 2N14 spectra indicate partitioning of this label between the membrane and water as a function of temperature (Wu & McConnell, 1974; Griffith & Jost, 1976). Both spin-labels partition in favor of the membrane hydrocarbon region as the temperature increases. These deviations in the spectra of 2N14 and 7N14 require that all spectral measurements of τ_c be considered only as motional indexes and not as absolute measurements of motion in the membrane (i.e., 2N14 motion may only be compared with a reference 2N14 spectrum, and in the same manner 7N14 motion is only compared relative to a reference 7N14 spectrum).

Leakage of Ferricyanide into Red Cells and Resealed Ghosts. Previous experiments by Morse (1977) showed that potassium ferricyanide may leak very slowly (on the order of days) into intact red blood cells. Small amounts of ferricyanide entering the cells would cause significant changes in the line width of the Tempamine signal which arises from the cell interior since ferricyanide is a very effective broadening agent for Tempamine (Morse, 1977; Keith et al., 1977c; Berg et al., 1979; Yager et al., 1979). This was examined for red cells and resealed ghosts by monitoring the mid-field line width of the 1 mM Tempamine signal as a function of time in the presence of 80 mM ferricyanide. Red blood cells were exposed to ferricyanide for 60 min, and no line broadening was observed (Figure 8). However, measurable line broadening of Tempamine occurred in the resealed ghosts after ~30 min. This suggests that the resealed ghosts are less capable of retaining ferricyanide impermeability than the red cells. Nevertheless, ferricyanide leakage was not considered a problem since both the red cells and resealed ghosts were exposed to ferricyanide for less than 15 min in all of the experiments described here.

Discussion

Our results have shown (1) that Tempamine motion in the aqueous interior of red blood cells and resealed ghosts at pH 7.5, 320 mosm, and 25 °C is hindered by a factor of 5 and 2.5, respectively, compared to bulk water, (2) that Tempamine is not bound detectably to any red blood cell components, (3) that Tempamine is randomly distributed throughout the interior of both cells and resealed ghosts, and lastly (4) that Tempamine motion within the cells responds to changes in cell size and temperature.

Factors Restricting the Motion of Tempamine in Intact Red Blood Cells. A number of mechanisms could account for our results. One is that Tempamine binds to hemoglobin during some part of its excited state, which is unlikely in view of the data presented in the Results. More likely are the possibilities that Tempamine rotational diffusion and hence Tempamine motion is restricted in some way by the presence of hemoglobin or that the environment sampled by Tempamine is actually more viscous than bulk water.

Water inside an intact red blood cell is never very far away from hemoglobin. If hemoglobin is taken to be a cylinder 57 Å in diameter with a center-to-center distance of 95 Å at 5 mM (Riley & Herbert, 1950), the approximate concentration of hemoglobin in the red blood cell under isoosmotic conditions, then there is a 38-Å channel of water between hemoglobin molecules. Over the range of 200–500 mosms, the range we studied, this distance can vary ~25%. Perutz (1978) determined that there should be a layer of water one molecule thick around each hemoglobin, although this has been questioned by Gary-Bobo & Solomon (1968). Thus, the aqueous environment inside the intact red blood cell is characterized by water in close association with hemoglobin. Since Tempamine can sample bound water (Keith et al., 1977b), Tempamine would be expected to give information about the average microviscosity of the cell interior. Considering the lifetime of the excited state of Tempamine (T_1

estimated at 10^{-7} s from line width data), Tempamine would move ~58 Å in this time in a medium with a bulk viscosity of 5 cP. Changes in the relative amounts of "free" and "bound" water as a function of osmolarity and pH (Perutz, 1978) could thus easily account for the variation in relative immobility of Tempamine in the red cell interior.

It is also possible that the water channels in packed hemoglobin could restrict the diffusion of Tempamine. Keith et al. (1977c) have shown that the spin-label 4-oxo-2,2,6,6-tetramethylpiperidinyl-*N*-oxy shows restricted motion in the pores of beads used for gel filtration. Smaller pore diameters restricted spin-label motion more than did larger pore diameters. Swelling and shrinking the red blood cells by osmotic mechanisms would cause a change in the size of the channel or pores between hemoglobin molecules and could result in a change of Tempamine motion. In the absence of information about the "state" of water in these pores, it is difficult to assign these changes in spin-label motion to more frequent collisions between the spin-labels and the "walls" of the channel or to an increased quantity of bound water inside the channels. We cannot at present make statements concerning the state of water inside the intact red blood cell on the basis of our results.

Factors Restricting the Motion of Tempamine in Resealed Red Blood Cell Ghosts. Interpretation of our results with resealed ghosts is actually less complex. Ghosts prepared by our methods are ~80–90% as large as intact cells and still retain their biconcave shape (Morse et al., 1978). Since Tempamine is randomly distributed within the lumen of these cells, the average Tempamine molecule inside a ghost is 2000–5000 Å away from any interior cell surface at any given time. To our knowledge, no soluble proteins exist within these resealed cells, although it is remotely possible that some of the inward-facing proteins such as spectrin could be released during the resealing process and trapped inside the cell. Electron microscopic evidence, however, shows no osmium-staining material in the cell lumen after resealing (Morse et al., 1978).

For rapid binding between Tempamine and the proteins of the cell membrane of resealed cells to give the observed results, Tempamine would have to be bound 1/2.5 of the time. If we assume a T_1 of 10^{-7} s, then Tempamine would only be able to diffuse 37 Å through a solution of 1 cP during the time it was not bound [10^{-7} s × (1/2.5) = 0.4 × 10^{-7} s]. If we interpret the ESR signal as arising entirely from Tempamine in rapid equilibrium with a binding site, this would require that all the Tempamine be within 37 Å of the membrane, which would lead to a local concentration of ~100 mM. We saw no ESR signals which were exchanged-broadened to this extent. A second value of the lifetime of the excited state of a spin-label (Dalton et al., 1975) is ~10^{-5} s, and calculations show that all Tempamine would have to be within 370 Å of the cell membrane. This is still a nonrandom distribution and still does not fit the line width data. Furthermore, if this were the only means by which Tempamine motion were hindered, entropy considerations would dictate that some Tempamine be present in the center of the lumen where it would tumble with a rotational correlaton time equivalent to that of bulk water. This is not observed in the results. We are therefore forced to conclude that the aqueous interior of the ghost is more viscous than bulk water. The mechanism by which Tempamine motion is restricted over these distances (2000–5000 Å) is unclear. Hypotheses concerning the long-range effect of surfaces on water order have been advanced (Drost-Hansen, 1973). Our data, however, cannot be construed as supporting these hypotheses to the exclusion of other hypotheses. We are presently performing experiments to illucidate the role of membrane proteins and lipids on the motion of Tempamine within the resealed ghosts.

Influence of pH, Temperature, and Osmotic Strength on 2N14 and 7N14 Motion. The spin-label 2N14 probes the membrane close to the membrane–water interface, while 7N14 probes the hydrophobic interior of the membrane (Morse et al., 1975). The parameter τ_c is used to measure the rotation of these spin-labels, but comparisons of motion may only be made for the same spin-label in the red blood cell [for an excellent review of these difficulties, see Schreier et al. (1978)]. This is because there are some additional contributions to the spectra which come, in some cases of 2N14, from the partitioning of the spin-label into the aqueous phase and/or its motional anisotropy and, in the case of 7N14, from motional anisotropy at low temperature. In our case, this serves to obfuscate the measurement of correlation time. For the 2N14 spectra, we make use of the fact that the high-field line is split sufficiently well so that the initial part of the hydrocarbon signal, which occurs downfield relative to the aqueous signal, does not overlap the aqueous signal. Our measurement of correlation time for 2N14 is made by taking only the height of the hydrocarbon line relative to the base line and multiplying by 2 to get the assumed height of the hydrocarbon line in its entirety. Although 2N14 shows partitioning in phosphatidylcholine vesicles (Morse, Clarkson, and Lusczakoski, unpublished experiments), the spectra of 2N14 in Figure 7 also suggest that some ordering of the probe occurs in the membrane (Gaffney & McConnell, 1974). This means that measurement of τ_c is imprecise in the absence of computer simulation techniques (Polnaszek & Freed, 1975).

In the case of 7N14, the measurement of rotational correlation time is less accurate at lower temperature since the spin-label tumbles in a somewhat anisotropic manner. Analysis of the molecular structure and hyperfine coupling constant suggests that 7N14 is oriented with the nitroxide z axis approximately perpendicular to the plane of the membrane. The spectral characteristics of spin-labels with this orientation have been analyzed extensively (Seelig, 1976). Analysis of the 7N14 spectra suggests that its orientation is not very rigid at low temperatures and that it has a considerable amount of wobble about the z axis.

The measurement of rotational correlation time for 2N14 and 7N14 as used here is best described as a relative motional index. In this sense, it is a very reproducible and precise measurement, but it does not give information about the absolute motion of 2N14 and 7N14 in the membrane. The spectra of these spin-labels are, however, sensitive to changes in membrane fluidity and accurately report these changes (Morse et al., 1975). Thus, the measurement of rotational correlation time gives important information about the state of the interior of the red blood cell membrane under the conditions described in this paper.

Wide ranges in pH and osmolarity which clearly affect the motion of Tempamine within the red blood cell aqueous interior affect neither the polar region of the membrane nor its hydrophobic interior as determined by 2N14 and 7N14, respectively. Temperature, on the other hand, does alter the membrane, as indicated by changes in spin-label motion, but these changes are essentially similar to those in bulk lipids (Morse, Clarkson, and Lusczakoski, unpublished experiments). This suggests that changes in the internal viscosity of red blood cells which are induced by variations of pH, osmolarity, or temperature are not in response to changes in those membrane regions accessible to 2N14 or 7N14. However, we cannot rule

out the possibility that conformational changes may be occurring in the membrane proteins or in those lipid regions of the membrane which are not sampled by 2N14 or 7N14.

Comparison of Data of Red Blood Cell Viscosity Obtained by ESR and Other Methods. Dintenfass (1968) has also studied the internal viscosity of the red blood cell as a function of osmotic strength and pH, but his results were obtained by measuring the shear or flow of red cells through capillary tubes. He found that crenated cells have a viscosity which is ~10 times greater than swollen cells and that the viscosity decreases by at least 2 orders of magnitude from pH 7.5 to 6.0. Our results on internal microviscosity as a function of osmotic pressure are in general agreement with Dintenfass, but our data on pH are not. The internal viscosities reported by Dintenfass were calculated from the rate of shear for solutions of blood, and the contribution of the membrane surrounding the red cell was not considered. As Dintenfass points out, his technique cannot distinguish between a fluid interior with a rigid membrane and a fluid membrane with a rigid interior. By use of electron spin resonance, the motion of spin-labels in the membrane and inside the cell can be studied on an individual basis. Nonetheless, it is the completely different nature of the measurements which accounts for the variation between our results and those of Dintenfass. Dintenfass is measuring the rheologic properties of a highly concentrated red cell suspension, whereas we are measuring the microviscosity as inferred from the motion of spin-labels which are physically inside the red blood cell during the time of measurement.

Conclusions

Our evidence indicates that both hemoglobin and the red cell membrane influence the rotational motion of Tempamine in the internal aqueous space of red blood cells and resealed ghosts, such that the microviscosity inside these cells is significantly greater than bulk water. Since this is not due to the binding of Tempamine to any intracellular or membrane component, it appears that the internal aqueous space is altered somehow by the presence of a membrane. The mechanism by which this occurs is not presently known or understood. We find that "fluidity" changes in the membrane under conditions similar to those of Dintenfass (1968) are not observed. This suggests that his results for untreated cells are solely due to changes in the hemoglobin. Our range of $\eta\mu$ for pH changes between 6 and 10 is much less than he observed. However, we have not measured red blood cell membrane fluidity under conditions of shear, and this must be taken into account when comparing his data to ours.

References

Beaudoin, A. G., & Mizukami, H. (1978) *Biochim. Biophys. Acta 532*, 41–47.

Berg, S. D., Lusczakoski, D., & Morse, P. D., II (1979) *Arch. Biochem. Biophys. 194*, 138–148.

Cooke, R., & Kuntz, I. D. (1974) *Annu. Rev. Biophys. Bioeng. 3*, 95–126.

Dalton, L. R., Coffey, L. H., Dalton, L. A., Robinson, B. H., & Keith, A. D. (1975) *Phys. Rev. A 11*, 488–498.

Dintenfass, L. (1968) *Haematologia 2*, 19–35.

Dix, J. A., Kivelson, D., & Diamond, J. M. (1978) *J. Membr. Biol. 40*, 315–342.

Drabkin, D. L., & Austin, J. H. (1932) *J. Biol. Chem. 98*, 719–733.

Drost-Hansen, W. (1973) *Ann. N.Y. Acad. Sci. 204*, 100–105.

Gaffney, B. J., & McConnell, H. M. (1974) *J. Magn. Reson. 16*, 1–28.

Gary-Bobo, C. M., & Solomon, A. K. (1968) *J. Gen. Physiol. 52*, 825–853.

Griffith, O. H., & Jost, P. (1976) in *Spin Labeling Theory and Applications* (Berliner, L., Ed.) pp 454–519, Academic Press, New York.

Haak, R. A., Kleinhans, F. W., & Ochs, S. (1976) *J. Physiol. (London) 263*, 115–137.

Hanahan, D. S. (1973) *Biochim. Biophys. Acta 300*, 319–340.

Hazlewood, C., Ed. (1973) *Ann. N.Y. Acad. Sci. 204.*

Henry, S. A., Keith, A. D., & Snipes, W. (1976) *Biophys. J. 16*, 641–654.

Keith, A. D., & Snipes, W. (1974) *Science 183*, 666–668.

Keith, A., Bulfield, G., & Snipes, W. (1970) *Biophys. J. 10*, 618–629.

Keith, A., Pollard, E. C., Snipes, W., Henry, S. A., & Culbertson, M. R. (1977a) *Biophys. J. 17*, 205–212.

Keith, A. D., Snipes, W., & Chapman, D. (1977b) *Biochemistry 16*, 634–641.

Keith, A. D., Snipes, W., Mehlhorn, R. J., & Gunter, T. (1977c) *Biophys. J. 19*, 205–218.

Kivelson, D. (1960) *J. Chem. Phys. 33*, 1094–1106.

Lowry, O. H., Rosebrough, H. J., Fahr, L., & Randall, R. S. (1951) *J. Biol. Chem. 193*, 265–275.

Morse, P. D., II (1977) *Biochem. Biophys. Res. Commun. 77*, 1486–1491.

Morse, P. D., II, Ruhlig, M., Snipes, W., & Keith, A. D. (1975) *Arch. Biochem. Biophys. 168*, 40–56.

Morse, P. D., II, Simpson, D., Mizukami, H., Lusczakoski, D., & Palazzo, R. (1978) *J. Supramol. Struct. S2*, 206.

Perutz, M. F. (1978) *Science 201*, 1187–1191.

Polnaszek, C. F., & Freed, J. H. (1975) *J. Phys. Chem. 79*, 2283–2306.

Riley, D. D., & Herbert, D. (1950) *Biochim. Biophys. Acta 4*, 374.

Rozantsev, E. G. (1970) *Free Nitroxyl Radicals*, pp 229–231, Plenum Press, New York.

Schreier, S., Polnaszek, C. F., & Smith, I. C. P. (1978) *Biochim. Biophys. Acta 515*, 375–436.

Seelig, J. (1976) in *Spin Labeling Theory and Applications* (Berliner, L., Ed.) pp 373–407, Academic Press, New York.

Williams, J. C., Mehlhorn, R., & Keith, A. D. (1971) *Chem. Phys. Lipids 7*, 207–230.

Wu, S. H. W., & McConnell, H. M. (1974) *Biochemistry 13*, 847–854.

Yager, T. D., Eaton, G. R., & Eaton, S. S. (1979) *Inorg. Chem. 18*, 725–727.