J. Membrane Biol. 101, 57–65 (1988)

The Journal of
Membrane Biology

A Flow EPR Study of Deformation and Orientation Characteristics of Erythrocyte Ghosts: A Possible Effect of an Altered State of Cytoskeletal Network

Toshiharu Ito* and Hideo Kon

Laboratory of Chemical Physics, National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, Maryland 20892

Summary. Using the flow EPR technique, we investigated the resealed ghost deformability in shear flow and the effects of the altered state of cytoskeletal network induced by hypotonic incubation of ghosts. Isotonically resealed ghosts in the presence of Mg-ATP, in which alteration of cytoskeletal network is not effected, have smooth biconcave discoid shapes, and show a flow orientation and deformation behavior similar to that of erythrocytes, except that higher viscosities are required to induce the same degrees of deformation and orientation as in erythrocytes. The flow behavior of resealed ghosts is Mg-ATP dependent, and the shape of the ghosts resealed without Mg-ATP is echinocytic. In contrast, the ghosts resealed by hypotonic incubation show a markedly reduced deformability even with Mg-ATP present. Nonreducing, nondenaturing polyacrylamide gel electrophoresis (PAGE) of the low ionic strength extracts from hypotonically resealed ghosts reveals a shift of the spectrin tetramer-dimer equilibrium toward the dimers. In the maleimide spin-labeled ghosts, the ratios of the weakly immobilized to the strongly immobilized EPR intensities are larger in hypotonically resealed ghosts than in the isotonically resealed ghosts, indicating an enhanced mobility in the spectrin structure in the former. Photomicrographs of hypotonically resealed ghosts show slightly stomatocytic transformations. These data suggest that the shape and the deformability loss in hypotonically resealed ghosts are related to an alteration of the spectrin tetramer-dimer equilibrium in the membrane. Thus, the shift of the equilibrium is likely to affect the regulation of the membrane deformability both in normal and pathological cells such as hereditary elliptocytes.

Key Words erythrocyte ghost · deformability · spectrin equilibrium · EPR

Introduction

During the past decade, evidence has been presented for the crucial role played by the cytoskeletal system in human erythrocytes, including spectrin, actin, and the band 4.1 protein, in maintaining the structural stability and the cell deformability [13]. Recently, using a variety of experimental techniques, several workers have focused attention on the studies of resealed ghosts, instead of on the whole cell, because of the advantage in ghosts of being able to probe directly the intrinsic physical properties of the membrane system. Heath et al. [14] have shown by a laser diffraction method that the deformation of resealed ghosts under shear stress reflects the change of the membrane mechanical properties modified by various manipulations. Nash and Meiselman [29, 30], and Nash, Tran-Son-Tay and Meiselman [31] examined the effects of lysing and resealing conditions on the ghost volume and the amount of the residual hemoglobin. Using the micropipette aspiration and the flow-channel techniques, they investigated the membrane viscoelastic properties of resealed ghosts, and found that the extention recovery time is shorter than in erythrocytes, but it approaches the value of the whole cell when ghosts are incubated in hypotonic medium.

The effect of hypotonic incubation of ghosts on the motional state of the cytoskeletal protein segments was studied by Farmer, Harmon and Butterfield [8]. They showed that the intensity ratio of the weakly immobilized to the strongly immobilized EPR signal of the SH-specific spin label (2,2,6,6-tetramethyl-4-maleimidopiperidine-1-oxyl (MAL-6)) in ghost membranes increased as the result of hypotonic incubation, and explained the observation on the basis of an increased motional freedom of spectrin segments by a hypotonic treatment.

Previously, Liu and Palek [21] demonstrated that spectrin exists in tetrameric form in the native state, but tetramers are also in reversible equilibrium with dimers in the membrane, and that partial transformation of tetramers to dimers by hypotonic incubation decreases structural stability of Triton-insoluble membrane skeletons. Liu and Palek [22] also showed that in hereditary elliptocytosis (HE),

* *Present address:* Department of Biochemistry, Nippon Medical School, 1-1-5, Sendagi, Bunkyo-Ku, Tokyo 113, Japan.

spectrin is partially in dimeric form, and that this may be responsible for the low stability of HE membrane skeletons. Liu et al. [23] also attributed the thermal and mechanical instability of the cytoskeleton in hereditary poikilocytosis erythrocytes to defective spectrin dimer-dimer association. Furthermore, Tchernia, Mohandas and Shohet [34] related the elliptocytosis and a marked decrease in the membrane stability and deformability of HE erythrocytes to a deficiency of protein band 4.1.

In the present work, we seek to answer the question as to whether the resealed whole ghost deformation and orientation characteristics in flow are affected by similar hypotonic treatments, in which the equilibrium in spectrin is shifted toward the dimers. The method we utilize is the flow EPR technique in which the flow-induced change in EPR spectral shape of 5-doxyl stearate spin labels intercalated in the membrane lipid phase are measured [3–5, 17, 32, 33] as a function of the volume flow rate. In this method, the spin labels report the extent of macroscopic cell deformation and spatial orientations of the cells (ghosts) under the influence of shear stress, instead of reporting the microviscosity in the membrane as in the usual applications of the spin-label technique. It has been demonstrated previously that such flow-induced EPR changes are closely related to the ability of the cells to deform and orient in flow [17]. We combined the flow EPR measurements with parallel observations of ghosts at rest and in flow by photomicrography which revealed the difference in the flow behavior between the isotonically and hypotonically incubated ghosts.

Materials and Methods

Adenosine-5′-triphosphate (ATP), dextrans (mean mol wt 40,000 and 150,000) and glutaraldehyde were purchased from Sigma Chemical Co.; dextran T500 (mean mol wt 500,000) and dextran T70 (mean mol wt 70,000) were from Pharmacia, the spin labels, 2,2,6,6-tetramethyl-4-maleimidopiperidine-1-oxyl (MAL-6) and 5-doxyl stearic acid were purchased from Aldrich. All other reagents were of analytical grade.

Preparation of Spin-labeled Ghost

Heparinized whole blood (heparin 20 units/ml) was obtained from healthy adult volunteers and was used within 24 hr. The blood was centrifuged at 2000 × *g* for 5 min. The plasma and buffy coat were thoroughly discarded by aspiration. Remaining packed erythrocytes were washed three times with 5 mM sodium phosphate buffer (pH 7.4) containing 145 mM NaCl, 5 mM KCl, and 10 mM D-glucose (medium I). Spin labeling of washed erythrocytes was performed as follows: 25 μl of ethanol solution of 12-doxyl stearic acid label (0.042 mg) was placed in a plastic test tube and was evaporated to dryness in a stream of N_2 gas to make a thin film of the label, to which were added 1.5 ml of packed erythrocytes (Ht > 90%) and an equal volume of medium I. The mixture was incubated at 37°C for 10 min with gentle shaking. The spin-labeled erythrocytes were washed once with medium I, and lysed in 20 volume of 5 mM sodium phosphate buffer (pH 8.0) at 0°C for 5 min, centrifuged at 27,000 × *g* for 10 min at 0°C, followed by washing once with the same buffer at 0°C. Resulting packed ghost was resuspended in resealing buffer (pH 7.4) containing 5 mM sodium phosphate, 4 mM $MgSO_4$, 0.5 mg/ml ATP and either 150 or 30 mM NaCl, respectively, for isotonic (295 mOsm) or hypotonic (82 mOsm) resealing. The ghost suspension was incubated at 37°C for 1 hr, followed by cooling in an ice bath for 5 min and centrifuged at 27,000 × *g* for 5 min. Finally, the packed ghosts resealed in the respective osmolality were suspended in buffered saline having the same osmolality and without $MgSO_4$ and ATP, and made up to 2 ml (termed resealed spin-labeled ghost suspension). No difference in shape or size was detected in the spin-labeled compared with the nonlabeled ghosts.

MAL-6 Spin-labeling of Ghosts

Washed packed erythrocytes (1.5 ml) were lysed in 30 ml of 5 mM phosphate buffer (pH 8.0) at 0°C followed by washing once with the same volume of buffer at 0°C. After centrifugation at 27,000 × *g* for 10 min at 0°C, packed ghosts were resuspended in 5 mM sodium phosphate buffer (pH 8.0) containing 0.02 mg/ml MAL-6, and were incubated for 12 hr. The temperature was kept at 0°C throughout the whole process. Ghosts were then centrifuged at 27,000 × *g* for 15 min and washed five times with 5 mM sodium phosphate buffer at 0°C. The pellet obtained was suspended in resealing buffer solutions (5 mM sodium phosphate, pH 7.4, 4 mM Mg/SO_4 and 0.5 mg/ml ATP, added with NaCl to adjust the osmolality to 295 or 82 mOsm), and was incubated at 37°C for 1 hr. Ghosts were then centrifuged at 27,000 × *g* for 5 min, and were used for EPR measurements.

Measurements of Ghost Deformability

Resealed spin-labeled ghost suspension (2 ml) was gently mixed into 3 ml of a medium containing 20% dextran T500, 5 mM sodium phosphate (pH 7.4), and 140 mM NaCl (isotonic) to make up a suspension having 12% dextran T500, 5 mM sodium phosphate (pH 7.4) and 2.8 × 10^9 cells/ml at 295 mOsm (isotonic) suspension. For hypotonically resealed ghost, the NaCl concentration was reduced to 10 mM. In either medium, the isotonic (295 mOsm) or hypotonic (82 mOsm) osmolality was maintained in the presence of dextran. The viscosity of both media was 31 cp.

To measure the deformability of resealed ghosts in shear flow, the suspension was driven by a computer-controlled syringe drive through a flat quartz cell having a 0.27 ± 0.01 mm wall-to-wall gap, 50 mm length, 8 mm width [18]. The flat cell was oriented in the EPR cavity resonator so that the flat surfaces were perpendicular to the magnetic field. The volume flow rate was increased linearly with time from 0 to 0.1 ml/sec during the measurement. During the flow, the magnetic field was held fixed at about 3370 gauss, where the maximal EPR spectral change (Δh) due to flow is expected. The field intensity for the maximal spectral change remains the same for a given spin label regardless of the type of specimens.[1] The normalized spectral change ($\Delta h/h$), where h is the maximal spectral amplitude measured in

[1] For a different spin label, e.g. 5-doxyl-phosphatidylcholine, the field must be reset for a new difference maximum. The set field is not susceptible to instrumental instabilities.

the absence of flow (Fig. 1), was plotted *vs.* the volume flow rate and was used as an index to assess the degree of the cell deformation and orientation in shear flow [17, 32, 33]. In this experimental condition, the volume flow rate of 0.1 ml/sec corresponds to the wall shear rate of 1028.8/sec. All EPR spectra were measured at room temperature, using a Varian Model E-109 X-band EPR spectrometer with 100 kHz field modulation.

Spectrin Extraction and Nonreducing Nondenaturing PAGE

Spectrin extraction in a low ionic strength buffer solution was performed according to the method by Liu and Palek [21]. Nondenaturing polyacrylamide gel electrophoresis (PAGE) of extracted spectrin was carried out with 2.3% polyacrylamide-0.3% agarose composite gels following the procedures of Liu et al. [23]. Gels were stained with 0.1% Coomasie Brilliant Blue R-250 solution and were analyzed by an LKB soft laser densitometer (Model SL-504).

Morphological Examinations

As ghosts are hardly visible even in the suspending medium of 12% dextran T500 solution, all morphological examinations of ghosts were performed under a Reichert light microscope equipped with a dark-field condenser. The combination of a dark-field optics and a phase-contrast objective lens gave greatly improved contrasts. Observations of flowing ghosts were made by using a specially constructed glass flow cell having flow space dimensions that closely approximate the size of the EPR quartz flow cell, except for the gap (0.22 ± 0.01 mm). The flow rate in this cell was adjusted accordingly to obtain approximately the same wall shear rate as in the flow EPR experiments. The EG&G FX-33C-15 xenon low pressure flash tubes were used as the light source operating at 300 to 500 V. The flash duration was estimated to be ca. 16 μsec. The microscope was focused at 10 μm below the upper surface of the flow cell. Kodak Recording Film or Kodak Tri-X Pan Film was used in photomicrography.

Results and Discussion

EPR Spectra of Flowing Cells

The flow-induced EPR spectral change is illustrated in Fig. 1 with 5-doxyl stearate-labeled erythrocytes as an example. It is known that the long-chain fatty acid labels are intercalated in the lipid phase with the long axes aligned approximately perpendicularly to the membrane surface [24]. In the absence of flow, when the cells are randomly oriented in suspension, the EPR spectrum of 5-doxyl stearate spin labels shows typical, strongly immobilized features (Fig. 1, dotted line). When deformable cells are in a flow under shear, the two outer peaks in the spectrum increase and the center peak decreases in intensity as the flow rate is increased (Fig. 1, solid line), indicating that more spin labels are oriented with their long axes in the direction of the magnetic field. Briefly, this is due to the fact that cells in

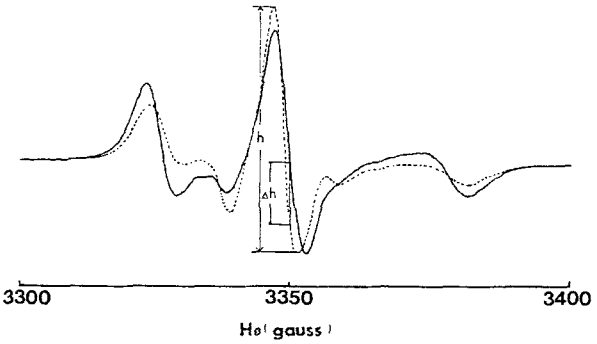

Fig. 1. A typical EPR spectrum of spin-labeled erythrocytes observed in the absence (. . .) and presence (—) of flow. Hematocrit 35%, extracellular dextran concentration 7.7%; h indicates the largest peak-to-trough height in the spectrum observed at rest, and Δh the maximal difference between the two spectra at 3370 gauss

shear flow are elongated to flattened ellipsoidal shapes with their flat surfaces aligned at various angles to the flow channel wall [10, 11, 17], which is oriented perpendicularly to the magnetic field. Consequently, more fatty acid spin labels on average are aligned by the shear flow with the long axes toward the magnetic field direction than in completely random cell orientations at rest or in tumbling cells. The simulation of EPR spectral shapes at various flow rates, taking into account variations in the degree of elongation and orientation due to the flow velocity distribution in the channel, has demonstrated the validity of the above model [17]. As the whole cell deformability is reduced for various reasons, the orientation becomes unsteady and cells eventually start tumbling [12] resulting in decreased EPR spectral change ($\Delta h/h$). Thus the flow EPR spectral shape reflects the average deformation and orientational state of flowing cells. For the purpose of comparing deformation/orientation characteristics, $\Delta h/h$ is plotted against the volume flow (or shear) rate; the higher the $\Delta h/h$, the greater the degree of cell deformation and orientation in flow. References [3–5, 17, 18, 32, 33] describe further details and applications of the method.

Effects of Preparatory Procedures

In ghost preparations, the EPR flow behavior was critically influenced by the preparation procedures. In Fig. 2, it is shown that the presence of both Mg^{2+} and ATP in isotonic resealing media enhances $\Delta h/h$ over the case added with ATP alone or neither. The difference in $\Delta h/h$ was more marked in the low flow-rate region, suggesting that the lower $\Delta h/h$ in the absence of Mg-ATP may be mostly due to some change in the membrane properties or the morphological reason [25–27]. That this indeed is the case

was shown by the echinocytic shapes of the majority of the isotonically resealed ghost prepared without Mg-ATP, while addition of Mg-ATP restored the biconcave discoid morphology (Figs. 3*a,b*). The lower $\Delta h/h$ in the absence of Mg-ATP was evidently due to the shape factor as was previously observed in ATP-depleted erythrocytes or in the echinocytosis caused by various reagents [25–27].

The flow behavior of ghost preparations was affected also by the duration of resealing incubation; when the ghosts, resuspended in a Mg-ATP-containing medium after lysis at 0°C, were immediately subjected to flow EPR measurements, $\Delta h/h$ was uniformly lowered over the entire range of the flow rate compared with those incubated for a longer period of time (Fig. 4). Almost fully developed flow characteristics were obtained by 10-min incubation, after which only a slight enhancement was observed. The incompletely resealed ghosts showed irregular shapes with echinocytic features.

Even when incubated with Mg-ATP for a sufficient length of time at 37°C, the ghosts generally required a suspending medium with a much higher viscosity to give $\Delta h/h$ values comparable to those of normal erythrocytes under the same condition. For example, in erythrocytes suspended in 7% dextran 40 at 35% hematocrit, the $\Delta h/h$ values at the shear rate of 1000/sec normally fall within the range of 0.35 to 0.45 [32], while a similar ghost suspension yielded $\Delta h/h$ of less than 0.2 even in 12% dextran 40. Use of dextrans having higher mean molecular weight resulted in corresponding enhancements in $\Delta h/h$ as shown in Fig. 5. The osmolalities of all suspending media in these measurements were adjusted to 295 mOsm/kg as closely as possible to avoid the effect of a volume change on $\Delta h/h$. The flow EPR curves of ghosts observed in 12% dextran T500 suspension resemble those of the normal erythrocytes in that $\Delta h/h$ rises rapidly in the low flow-rate region followed by a more gradual increase toward the value of 0.4 to 0.5 at the shear rate of 1000/sec. Thus in the rest of the experiments, dextran T500 was used at 12% (wt/vol) in appropriate buffer solutions. The difference between ghosts and erythrocytes in their response to viscosity of the medium seems consistent with the observations by others that the flow channel and micropipette elongation methods yielded substan-

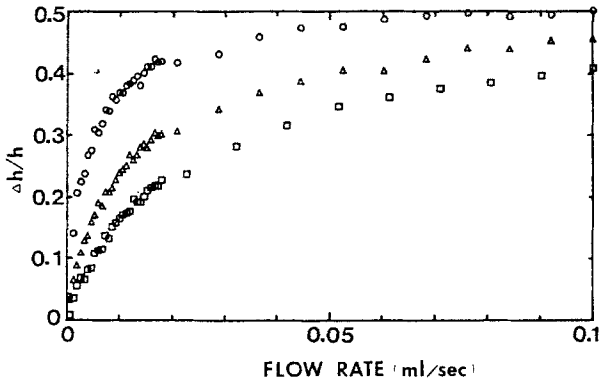

Fig. 2. Effect of Mg-ATP on deformation and orientation characteristics. Dextran T500 concentration 12%, cell number 2.8 × 10^9/ml; ghosts prepared from 5-doxyl stearate-labeled erythrocytes, were incubated in isotonic buffer solution containing 4 mM $MgSO_4$ and 0.5 mg/ml ATP (○), 0.5 mg/ml ATP only (△), or no $MgSO_4$-ATP (□). The flow behavior of ghosts incubated in an isotonic medium with 4 mM $MgSO_4$ alone was the same as that incubated in isotonic medium only

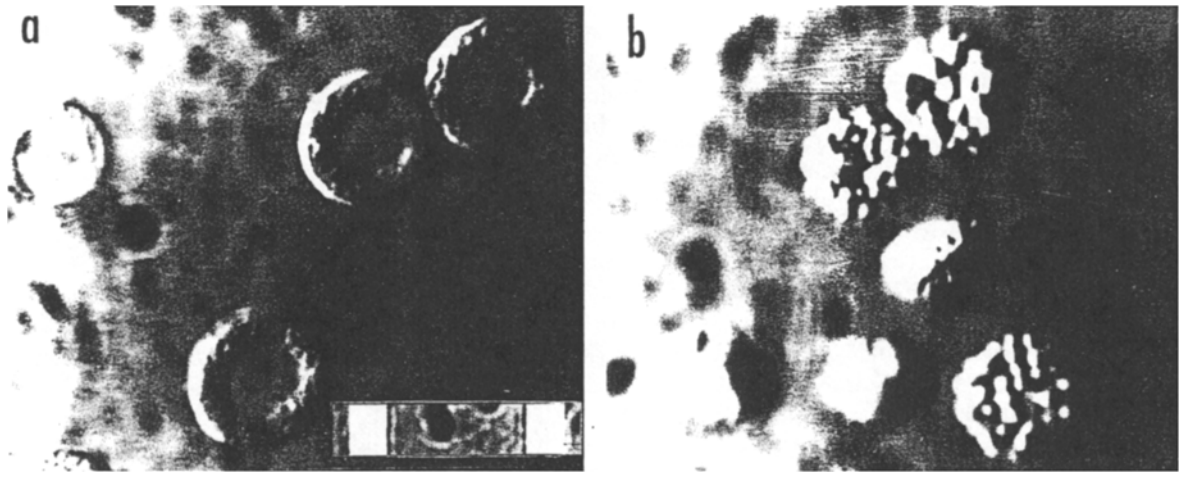

Fig. 3. Effect of Mg-ATP on the shape of isotonically resealed ghosts. (*a*) Ghosts incubated in isotonic buffer with Mg-ATP, and (*b*) without Mg-ATP. Ghosts were fixed in 1% glutaraldehyde solution at 0°C for 30 min. NIKON differential interference optics were used. The scale indicates 10 μm

tially higher values of the membrane elastic modulus, although the results by micropipette aspiration were not conclusive [31].

Other factors which affect the ghost flow behavior include the ionic strength and the dilution ratio in the lysis medium. We found that a lower ionic strength and/or a larger dilution ratio at lysis tends to suppress the $\Delta h/h$ values. The effect may be explained by partial loss of spectrin [15] and/or a possible structural rearrangement of spectrin [21, 23, 34].

Effect of Hypotonic Incubation

The optimal preparatory conditions thus found as described in Materials and Methods were applied to observe the effects of a hypotonic treatment on the deformation and orientation properties of ghosts in flow, which may be influenced by the shift of the tetramer-dimer equilibrium in spectrin toward the dimers [8, 21, 30].

The two preparations of ghosts from erythrocytes hypotonically lysed at 0°C in 5 mM phosphate buffer (pH 8.0) and resealed at 37°C in, respectively, hypotonic (82 mOsm) and isotonic (295 mOsm) phosphate-buffered saline with Mg-ATP were compared with respect to $\Delta h/h$ and the morphologies at rest and in flow.

The flow EPR curves of the two preparations were measured in dextran T500 and T70 as shown in Fig. 6. In these suspending media, the respective osmolality at the time of isotonic and hypotonic incubation was accurately maintained in the presence of dextran to eliminate any volume effects on the ghost deformability. The $\Delta h/h$ values were remarkably reduced in the hypotonically incubated ghosts in both dextran media, and the difference from the isotonic ghosts increased with the flow rate and were greater in dextran T500 than in T70. In hypotonically resealed ghosts, $\Delta h/h$ reached a plateau at a relatively low flow rate (ca. 0.01 to 0.02 ml/sec), indicating that the ability of the ghost to deform and orient came to a limit in an early stage of the flow. The isotonic ghosts, in contrast, showed a continuing increase in $\Delta h/h$ as the flow rate was increased.

Photomicrographically the ghosts in both preparations had a general appearance similar to those of biconcave erythrocytes, but the volumes measured by the Coulter counter method were approxi-

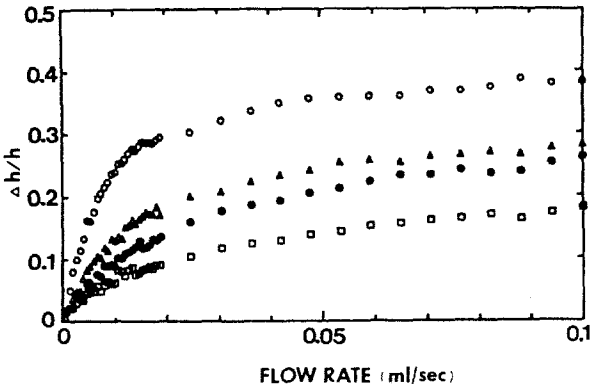

Fig. 5. Effect of the suspending medium viscosity on the flow EPR characteristics. Isotonically resealed ghosts were suspended in isotonic-buffered saline containing 12% dextrans. (○) T500 (31.0 cp), (△) dextran 150 (18.3 cp), (●) T70 (12.1 cp), (□) dextran 40 (8.1 cp)

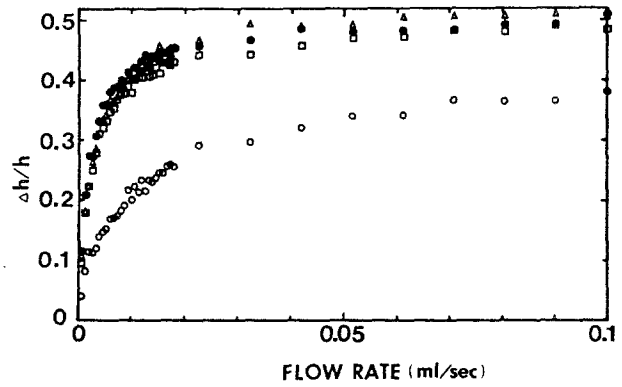

Fig. 4. Effect of duration of resealing incubation on ghost deformation and orientation characteristics. Dextran (mol wt 500,000) concentration 12%, cell number 2.8×10^9/ml; ghosts prepared by hypotonic lysis were incubated in isotonic buffer solution containing 4 mM $MgSO_4$ and 0.5 mg/ml ATP at 37°C for 0 (○), 10 (□), 30 (●) or 60 min (△)

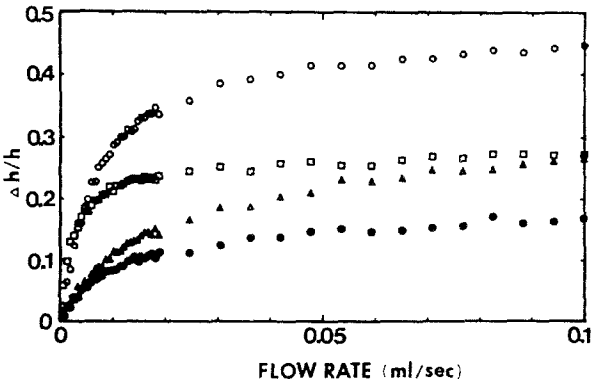

Fig. 6. Effect of osmolality of resealing medium on the flow EPR characteristics. Ghosts resealed in isotonic (295 mOsm) medium containing 4 mM $MgSO_4$ and 0.5 mg/ml ATP were suspended in isotonic (295 mOsm)-buffered saline containing 12% dextran T500 (○), or T70 (△). Ghosts resealed in hypotonic (82 mOsm) medium containing Mg-ATP as above were suspended in hypotonic (82 mOsm)-buffered saline containing 12% dextran T500 (□), or T70 (●)

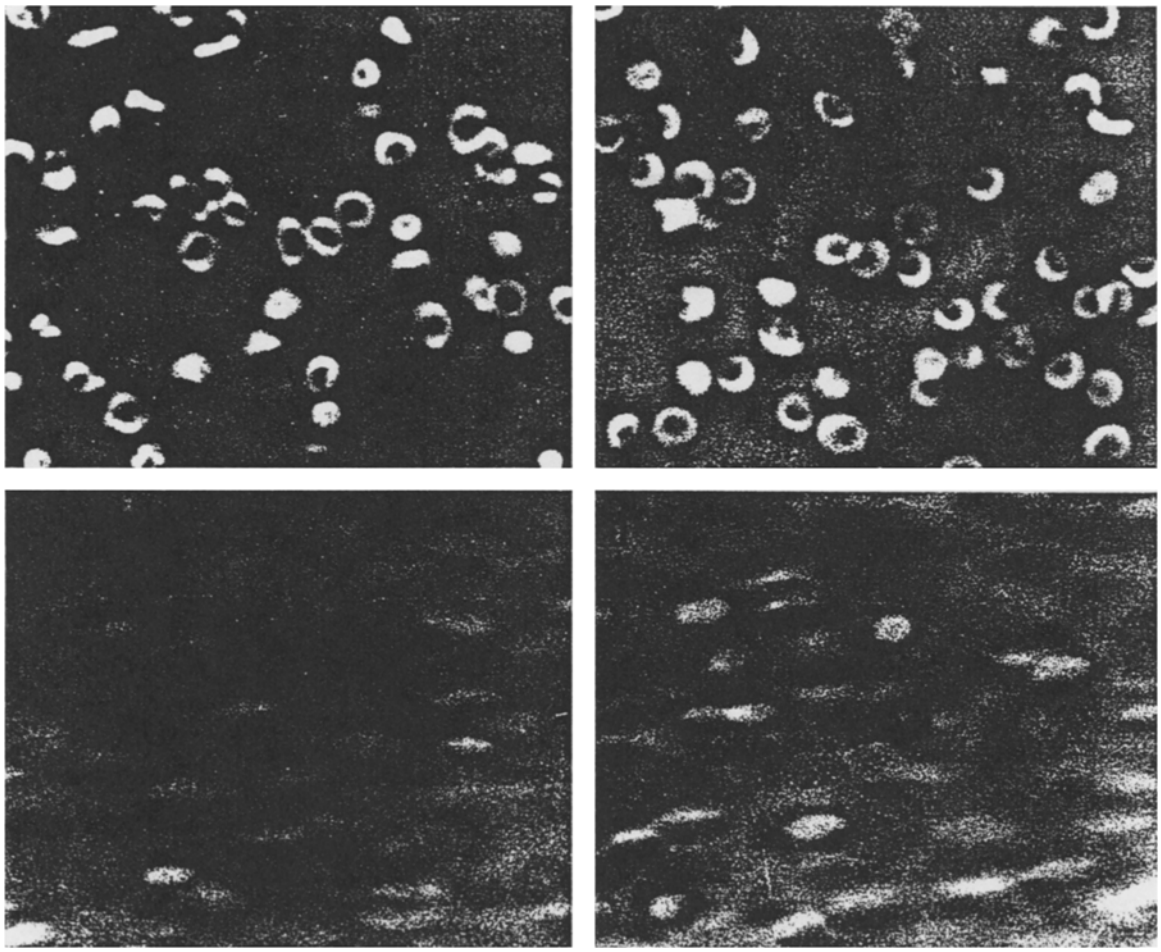

Fig. 7. Ghost shapes in the absence and presence of shear flow. Dextran T500 concentration 12%, cell number 2.1×10^8/ml; dark-field illumination was used. (*a*) isotonically resealed, no flow; (*b*) hypotonically resealed, no flow; (*c*) isotonically resealed, wall shear rate 1500/sec; (*d*) hypotonically resealed, wall shear rate 1500/sec. Patterns were virtually the same at the shear rate of 1000/sec

mately 60% of that of the average normal erythrocytes. The isotonically resealed ghosts showed smooth surface and biconcave shapes, while the hypotonically resealed ghosts showed slightly stomatocytic shape changes of stage I [2] (Figs. 7*a*,*b*). Both types of ghost contained 2 to 3% of the original hemoglobin. When flowing at a low flow rate (<0.005 ml/sec), both types of ghosts were partially oriented parallel to the flow cell wall with a slight deformation. Consistent with this observation, the $\Delta h/h$ *vs.* flow-rate curves of isotonic and hypotonic ghosts coincided in the low flow-rate region (Fig. 6). As the flow rate was increased beyond 0.005 ml/sec, the isotonic and hypotonic ghosts under shear stress were elongated and oriented with the long axes aligned along the direction of flow just as in erythrocytes, but the extent of apparent elongation in hypotonically incubated ghosts was on average always less than in the corresponding isotonic ghosts. Typical flow patterns at the shear rate of 1500/sec are compared in Figs. 7(*c*,*d*).

The spectrin dimer-to-tetramer ratios in isotonically and hypotonically resealed ghosts were estimated by nonreducing, nondenaturing polyacrylamide gel electrophoresis. The densitometric peak height ratios were found shifted by ca. 15% on average toward the dimers when ghosts were resealed in 30 mM NaCl phosphate buffer compared to those in 150 mM NaCl buffer. Resealing in 5 mM NaCl-buffered solution yielded dimer and tetramer peaks with almost equal heights. These trends which were repeatedly observed are qualitatively in good agreement with the results reported by Liu and Palek [21], and indicate that the fraction of dimeric spectrin indeed increased in the hypotonically incubated ghosts which also showed much decreased $\Delta h/h$ in flow.

A further comparison was made of the two

types of ghosts with respect to the motional state of the cytoskeletal proteins following Farmer et al. [8], using a sulfhydryl specific spin label MAL-6. It has been amply demonstrated that under the present experimental conditions, MAL-6 is bound covalently predominantly to spectrin and also to actin, bands 4.1, 2.1, and the cytoplasmic side of band 3, giving rise to a pair of EPR absorptions which represent, respectively, weakly and strongly immobilized state of the labeled sites in the proteins involved in the cytoskeletal network [1, 6, 7]. The ratio of the two absorption intensities has been shown to report reliably the motional states of the attached proteins.

In the present work, the concentration of the hypotonic resealing medium was made higher than in Farmer et al. [8], so that the preparation of ghosts for the MAL-6 label measurements were made under the same conditions as in the flow EPR experiments. The ratios of the weakly immobilized to the strongly immobilized (W/S) signal intensities of MAL-6 in the low-field EPR absorption were 3.01 ± 0.16 and 3.21 ± 0.22 in our experimental conditions in, respectively, isotonically and hypotonically incubated ghosts. A higher W/S ratio was interpreted by Farmer et al. [8] as an indication of an enhanced segmental freedom of motion in the cytoskeletal network as the result of an increased spectrin dimer fraction. The somewhat smaller W/S ratios we obtained compared to those by Farmer et al. [8] are partly due to the relatively higher NaCl (30 mM) concentration in our hypotonic incubation. This was verified by an observation that the W/S ratio increased with decreasing ionic strength in resealing, reaching ca. 5 at 5 mM NaCl concentration as reported by Farmer et al. [8]. A relatively higher NaCl concentration in our procedure was necessary to match the osmolality in the MAL-6 experiments to that of the flow EPR experiments in which the presence of dextran inevitably raised the lower limit of the hypotonicity. It was also noted that the presence of Mg-ATP generally lowered the W/S ratio. It is likely that Mg-ATP suppresses the freedom of segmental motion and stabilizes the cytoskeletal network in ghosts. Kansu, Krasnow and Ballas [15] demonstrated that there is a time-dependent release of spectrin from human erythrocyte membrane during the cell lysis in 5 mM phosphate buffer (no NaCl, Mg-ATP). If a similar leaching occurs during the low ionic strength resealing in the absence of Mg-ATP, the loss of spectrin may be irreversible, and this may cause an alteration in the cytoskeletal network and subsequently reduce the ghost deformability. Thus, a low ionic strength as well as the presence or absence of Mg-ATP appears to influence the freedom of segmental motion in ghost cytoskeletal proteins which play a crucial role in maintaining the deformation and orientation behavior of ghosts in flow. The precise mechanism for the effect of Mg-ATP is presently unclear.

We have shown that the hypotonically (82 mOsm) resealed ghosts in the presence of Mg-ATP exhibit behaviors distinct from those isotonically (295 mOsm) resealed in that (1) the $\Delta h/h$, a measure of ghost's ability to deform and/or orient in flow, is markedly lowered except in the low flow-rate region, (2) the MAL-6 EPR signals show an increased motional freedom in spectrin segments in agreement with the previous study, and (3) the ghost shape is slightly stomatocytic.

The change in the MAL-6 EPR signals by hypotonic incubation was explained by Farmer et al. [8] on the basis of an increased dimeric fraction in spectrin by hypotonic treatments. The reduced deformation/orientation ($\Delta h/h$) in hypotonic ghosts is also accompanied by a shift in the spectrin equilibrium. The reduced deformation/orientation ($\Delta h/h$) and the shift in dimer/tetramer ratio to the dimer side observed in hypotonic ghosts can be simply two parallel events with no cause and effect relation, and are connected to some undefined change in the cytoskeletal structure. On the other hand, it is interesting to enquire into a possible relation between the two observations.

First, the possibility of the observed stomatocytic morphology being the principal reason for the reduced $\Delta h/h$ in the hypotonic ghosts can be ruled out. The whole cell deformability is generally influenced by factors such as (1) a perturbation on the physical characteristics of the membrane system or morphology, (2) the surface area-to-volume relation, and (3) the ratio of the intracellular and the outer fluid viscosity [28]. It has been observed that an effect of the morphological changes on rheological characteristics (bulk viscosity, deformability) of the whole cell are most clearly observed in the low shear rate region and the effects tend to diminish as the shear rate is increased [26, 27]. In contrast, the internal viscosity and the surface area-to-volume ratio usually affect the flow behavior over the entire region of shear rate. These trends have been confirmed also by the present flow EPR technique [17, 32]. Therefore, if the stomatocyte I morphology of the hypotonically resealed ghosts is the cause of the observed decrease in $\Delta h/h$, the effect is expected to be greater in the low shear-rate region; however, the decrease in $\Delta h/h$ is rather small in the low shear region and becomes greater as the shear rate and the suspension viscosity is increased.

On the other hand, in the present experimental procedures for hypotonic incubation, there are no steps involved which may result in changes in the

area-to-volume relation or the internal viscosity.[2] In some procedures for ghost preparation [30, 31], the hypotonically lysed membrane at 0°C is equilibrated in a medium with an elevated salt concentration before raising the temperature to 37°C for resealing. The mean ghost volume changes during the period depending upon the salt concentration and the duration of the period [30]. In the present experiments, this step was eliminated by introducing prewarmed resealing buffer (isotonic or hypotonic) at the end of the lysis period, so that osmolality adjustment and resealing proceed simultaneously. The resealed isotonic and hypotonic ghosts thus obtained are virtually indistinguishable under microscope (Figs. 7*a,b*), except for the stomatocytic shape in the latter.

A tentative explanation for the markedly reduced $\Delta h/h$ in the hypotonically resealed ghosts may be that owing to the altered state of spectrin assembly, the orientation of the hypotonically resealed ghosts may be less steady, undergoing intermittent flippings. The $\Delta h/h$, which depends upon the population difference in spatial orientations of the spin label, should decrease to the extent that the ghosts tumble with a time constant greater than the EPR time scale (ca. 10^{-6} sec). Such tumbling motions are not expected to show in the flow photomicrography experiments. Nash et al. [31] have shown that a prolonged exposure of ghosts to a hypotonic medium in resealing causes a longer extension recovery time, which they attributed to an increased membrane viscosity rather than a reduced modulus of elasticity. An increased membrane viscosity could adversely affect tank-treading motion [9] in flow. It is possible, though not proven, that an altered state of spectrin may disturb the tank treading motion [35]. The relationship between steady orientation in flow and tank-treading of erythrocytes has been theoretically clarified [16].

Although the extent of shift in the spectrin equilibrium realized in the present experimental conditions is relatively small (ca. 15%), the observed effects on the flow behavior are quite evident, manifesting the possible sensitivity of the spectrin modification in regulating the flow characteristics.

We wish to thank Dr. Makio Murayama for his extensive help in photomicrography, and Dr. Michael Lieber for the useful discussions on ghost resealing.

[2] The relations of resealing conditions and the hole size in the membrane have been extensively studied by Lieber and Steck [19, 20]. The hole size expected in the present hypotonic resealing condition (30 mM NaCl, 37°C, 1 hr) precludes passage of high molecular weight dextrans.

References

1. Barber, M.J., Rosen, G.M., Rauckman, E.J. 1983. Studies of the mobility of maleimide spin labels within the erythrocyte membrane. *Biochim. Biophys. Acta* **732:**126–132
2. Bessis, M. 1973. Red cell shapes: An illustrated classification and its rationale. *In:* Red Cell Shape. M. Bessis, R. I. Weed and P. F. Leblond, editors. pp. 1–25. Springer, New York
3. Bitbol, M., Leterrier, F. 1982. Measurement of the erythrocyte orientation in a flow by spin labeling. I. Comparison between experimental and numerically simulated E.P.R. spectra. *Biorheology* **19:**669–680
4. Bitbol, M., Leterrier, F., Quemada, D. 1985. Measurement of the erythrocyte orientation in a flow by spin labeling. III. Erythrocyte orientation and rheological conditions. *Biorheology* **22:**43–53
5. Bitbol, M., Quemada, D. 1985. Measurement of the erythrocyte orientation in a flow by spin labeling. II. Phenomenological models for erythrocyte orientation rate. *Biorheology* **22:**31–41
6. Butterfield, D.A. 1982. Spin labeling in disease. *Biol. Mag. Res.* **4:**1–78
7. Butterfield, D.A., Markesbery, W.R. 1981. On the use of a piperidine maleimide spin label to investigate membrane proteins in erythrocyte membrane with reference to Huntington's disease. *Biochem. Int.* **3:**517–525
8. Farmer, B.T., II, Harmon, T.M., Butterfield, D.A. 1985. ESR studies of the erythrocyte membrane skeletal protein network: Influence of the state of aggregation of spectrin on the physical state of membrane proteins, bilayer lipids, and cell surface carbohydrates. *Biochim. Biophys. Acta* **821:**420–430
9. Fischer, T.M., Stöhr, M., Schmid-Schönbein, H. 1978. Red blood cell (RBC) microrheology: Comparison of the behavior of single RBC and liquid droplets in shear flow. *Am. Inst. Chem. Eng. Symp.*, Ser. No. 182. **74:**38–45
10. Goldsmith, H.L. 1971. Deformation of human red cells in tube flow. *Biorheology* **7:**235–242
11. Goldsmith, H.L., Marlow, J.C. 1972. Flow behavior of erythrocyte. I. Rotation and deformation in dilute suspensions. *Proc. R. Soc. London B* **182:**351–384
12. Goldsmith, H.L., Marlow, J.C. 1979. Flow behavior of erythrocytes. II. Particle motions in concentrated suspensions of ghost cells. *J. Colloid Interface Sci.* **71:**383–407
13. Haest, C.W.M. 1982. Interaction between membrane skeleton proteins and the intrinsic domain of the erythrocyte membrane. *Biochim. Biophys. Acta* **694:**331–352
14. Heath, B.P., Mohandas, N., Wyatt, J.L., Shohet, S.B. 1982. Deformability of isolated red blood cell membranes. *Biochim. Biophys. Acta* **691:**211–219
15. Kansu, E., Krasnow, S.H., Ballas, S.K. 1980. Spectrin loss during in vivo red cell lysis. *Biochim. Biophys. Acta* **596:**18–27
16. Keller, S.R., Skalak, R. 1982. Motion of a tank-treading ellipsoidal particle in a shear flow. *J. Fluid Mech.* **120:**27–47
17. Kon, K., Noji, S., Kon, H. 1983. Spin label study of erythrocyte deformability. III. Further characterization of electron spin resonance spectral change in shear flow. *Blood Cells* **9:**427–438
18. Kon, K., O'Bryan, E.R., Kon, H. 1985. Effect of the presence of hardened erythrocytes on deformation-orientation characteristics of normal erythrocytes in shear flow studied by the spin label method. *Biorheology* **22:**105–117

19. Lieber, M.R., Steck, T.L. 1982. A description of the holes in human erythrocyte membrane ghosts. *J. Biol. Chem.* **257:**11651–11659
20. Lieber, M.R., Steck, T.L. 1982. Dynamics of the holes in human erythrocyte membrane ghosts. *J. Biol. Chem.* **257:**11660–11666
21. Liu, S.C., Palek, J. 1980. Spectrin tetramer-dimer equilibrium and the stability of erythrocyte membrane skeletons. *Nature (London)* **285:**586–588
22. Liu, S.C., Palek, J. 1980. Decreased spectrin tetramer-dimer ratio and mechanical instability of membrane skeletons in hereditary elliptocytosis. *Clin. Res.* **28:**318a
23. Liu, S.C., Palek, J., Prchal, J., Castleberry, R.P. 1981. Altered spectrin dimer-dimer association and instability of erythrocyte membrane skeletons in hereditary pyropoikilocytosis. *J. Clin. Invest.* **68:**597–605
24. McConnell, H.M. 1976. Molecular motion in biological membranes. *In:* Spin Labeling. Theory and Applications. L.J. Berliner, editor. pp. 525–560. Academic, New York
25. Meiselman, H.J. 1977. Flow behavior of ATP-depleted human erythrocytes. *Biorheology* **14:**111–126
26. Meiselman, H.J. 1978. Rheology of shape transformed human red cells. *Biorheology* **15:**225–237
27. Meiselman, H.J. 1981. Morphological determinants of red cell deformability. *Scand. Clin. Lab. Invest.* (41 Suppl.) **156:**27–34
28. Mohandas, N., Clark, M.R., Jacobs, M.S., Shohet, S.B. 1980. Analysis of factors regulating erythrocyte deformability. *J. Clin. Invest.* **66:**563–573
29. Nash, G.B., Meiselman, H.J. 1983. Red cell and ghost viscoelasticity: Effects of hemoglobin concentration and in vivo aging. *Biophys. J.* **43:**63–73
30. Nash, G.B., Meiselman, H.J. 1985. Effects of preparative procedures on the volume and content of resealed red cell ghosts. *Biochim. Biophys. Acta* **815:**477–485
31. Nash, G.B., Tran-Son-Tay, R., Meiselman, H.J. 1986. Influence of preparative procedures on the membrane viscoelasticity of human red cell ghosts. *Biochim. Biophys. Acta* **855:**105–114
32. Noji, S., Inoue, F., Kon, H. 1981. Spin label study of erythrocyte deformability. I. Electron spin resonance spectral change under shear flow. *Blood Cells* **7:**401–411
33. Noji, S., Kon, H., Taniguchi, S. 1984. Spin label study of erythrocyte deformability. IV. Relation of ESR spectral change with deformation and orientation of erythrocytes in shear flow. *Biophys. J.* **46:**349–355
34. Tchernia, G., Mohandas, N., Shohet, S.B. 1981. Deficiency of skeletal membrane band 4.1 in homozygous hereditary elliptocytosis. *J. Clin. Invest.* **68:**454–460
35. Tran-Son-Tay, R., Sutera, S.P., Rao, P.R. 1984. Determination of red blood cell membrane viscosity from rheoscopic observations of tank-treading motion. *Biophys. J.* **46:**65–72

Received 25 June 1987; revised 29 September 1987