COLD SHOCK HEMOLYSIS IN HUMAN ERYTHROCYTES STUDIED BY SPIN PROBE METHOD AND FREEZE-FRACTURE ELECTRON MICROSCOPY

TSUNEO TAKAHASHI,* SUMIHARE NOJI,* ERIC F. ERBE,‡ RUSSELL L. STEERE‡ AND HIDEO KON*
*Laboratory of Chemical Physics, National Institute of Arthritis, Diabetes, and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, Maryland 20892; and ‡Plant Stress Laboratory, A.R.S., U.S. Department of Agriculture, Beltsville, Maryland 20705

ABSTRACT When human erythrocytes are osmotically stressed or chemically treated, they hemolyze on cooling below 10°C (called cold shock). We have studied the effects of osmotic stress and cooling on the state of membrane by the spin-probe method and freeze-fracture electron microscopy. At room temperature, the membrane fluidity detected by 12-doxyl stearate spin probe showed a steady decrease with osmolality in hypertonic NaCl solutions up to 900 mOsm/kg, above which it remained unchanged. In hypertonic sucrose solutions, the electron paramagnetic resonance spectra showed an additional pair of absorptions, indicating development of regions, in the membrane, further immobilized than in NaCl solutions. Mobility of a cholesterol analogue probe, androstane, did not show change by hypertonicity, but the spectral intensity dropped at 1,200 mOsm/kg, probably due to formation of loose aggregates in the cholesterol phase. On cooling the osmotically stressed cells in NaCl solution, the isotropic rotational correlation time vs. inverse temperature plot of 12-doxyl stearate probe exhibited a step-wise discontinuity at ~10°C, suggestive of a drastic transition in the state of the membrane. At about the same temperature, the freeze-fracture pattern of osmotically stressed cells revealed the development of large wrinkles and aggregation of membrane particles, in contrast to the case of the cells in isotonicity. Significance of these findings in understanding cold shock hemolysis is discussed.

INTRODUCTION

Cold shock is generally defined as injury to cells occurring during rapid cooling. It has been reported to occur in diverse group of cells, such as some species of plant cells, spermatozoa, bacteria, embryos, granulocytes, and erythrocytes (1, 2). In erythrocytes, cold shock is observed under two circumstances. In the first, cells suspended in hypertonic solutions are hemolyzed when the cells are cooled (3, 4). The second type of cold shock is observed when cells pretreated with a toxin of microorganisms are cooled in an isotonic medium (5).

Although many investigators have studied this phenomenon, the molecular mechanism of cold shock remains unclear. Lovelock, who discovered hemolysis in hyperosmotically stressed human erythrocytes, speculated that it results from partial release of phospholipid and cholesterol from the membrane during osmotic stress (3, 4). However, subsequent reports (6, 7) disputed the correlation. Also, several authors surmised involvement of the spectrin-actin system in cold shock hemolysis (8–10).

Previously, we have suggested that it is crucial for the cell to decrease in volume to a certain level to become susceptible to cold shock, and have located the onset of hemolysis at ~10°C (11). In a continuing effort, in the present study we have investigated cold shock hemolysis from the viewpoint of thermotropic structural transition in cell membranes using the electron paramagnetic resonance (EPR) spin probe technique and freeze-fracture electron microscopy. Nunes, who has recently reported on the effect of osmolality (300–3,000 mOsm/kg) on membrane fluidity at room temperature by using a similar spin probe technique, did not detect any change in membrane fluidity throughout the range (12).

However, we will show that EPR parameters do change as a function of osmolality at room temperature, and also that when the temperature is lowered below 10°C, the rotational correlation time of a spin probe shows a discontinuity, which is suggestive of a transition in the state of cell membrane undergoing cold shock. At about the same temperature, marked wrinkles are observed in the freeze-fractured inner surface of the membrane. The observations provide strong evidence for a major structural rearrangement involving the membrane cytoskeletal system in osmotically stressed erythrocytes.

Dr. Takahashi's present address is Transplantation Laboratory, American Red Cross Blood Services Laboratories, Bethesda, MD 20814
Dr Noji's present address is Department of Biochemistry, Okayama University Dental School, 2-5-1 Shikata-cho, Okayama, 700 Japan
Correspondence should be addressed to H. Kon, Room B1-14, Building 2, NIH, Bethesda, MD 20892

MATERIALS AND METHODS

Preparation of Erythrocyte Suspensions

Whole human blood was collected in full units with citrate phosphate dextrose anticoagulant. Platelet-rich plasma was removed within 2 h after collection by centrifugation at 2,600 *g* for 3.25 min. Packed erythrocytes were washed three times with isotonic phosphate-buffered saline (PBS, 150 mM NaCl, 6.7 mM KH_2PO_4-Na_2HPO_4 buffer, pH 7.4) before use. Hypertonic solutions were made by adding NaCl or sucrose to the isotonic PBS. The osmolality was measured on a freezing-point osmometer.

Measurements of Cold Shock Hemolysis

Ten μl of packed cells were suspended in 2 ml of hypertonic solution in 13 × 100 mm glass test tubes at the selected starting temperature. After incubation in a temperature controlled bath, the cells were cooled by immersing the test tubes in ice water. The cooling rate was ~60°/min between 15 and 0°C. The temperature was measured with a digital thermometer. The extent of hemolysis was determined by measuring hemoglobin release into the suspending solution. Undamaged cells were removed by centrifugation at 1,000 *g* for 5 min. The supernatant was diluted with Drabkin's solution (Fisher Scientific Co., Pittsburgh, PA), and the optical absorbance of cyanomethemoglobin was measured at 540 nm. Complete hemolysis was determined with 10 μl of red cells hypotonically lysed in 2 ml of dilute Drabkin's solution. The hemolysis was also measured by means of light scattering at 590 nm as described before (11). The device was attached to a cryomicroscope so that the onset and the amount of hemolysis could be measured simultaneously (cooling rate: 9°C/min).

Incorporation of Spin Probes

Spin probes, 5-, 12-, and 16-doxyl stearic acid and androstane were purchased from Syva Corp. (Palo Alto, CA). Packed erythrocytes (hematocrit 90–92%) were incubated in a flask coated inside with a thin film of a spin probe for 10 min at 37°C. The hematocrit was adjusted to 35% for EPR measurements by adding an appropriate amount of various hypertonic PBS solutions. The final concentration of spin probe in suspension was ~6.5×10^{-5} M, at which level no morphological change or hemolysis was observed.

EPR Measurements

EPR measurements were made in silicone-coated 50 μl capillary tubes on an X-band EPR spectrometer (model CE109; Varian Associates Inc., Palo Alto, CA). The amplitude of 100 KHz field modulation was set at 2 Gs, and the microwave power was kept at 40 mM, at which level no saturation effect was detected. The capillary containing the sample was surrounded by a single-jacketed quartz dewar insert, through which dry nitrogen gas of constant temperature (24 ± 0.05°C) was passed at a rate of 6 liters/min.

Freeze-fracture Electron Microscopy

Erythrocytes suspended in isotonic (300 mOsm/kg) or hypertonic (1,800 mOsm/kg) NaCl were placed in a standard 24K gold complementary freeze-fracture holder held at 24°C. The loaded specimen holder was then placed in a petri-dish that was immersed in an ice-water bath. When the holder temperature reached 10° or 0°C, the holder was plunged into Freon-22 that had been cooled at −120°C. These holders were then transferred to a Denton DV-502 freeze-fracture apparatus (14), and fractured at −150°C. Without etching, samples were shadowed with platinum followed by carbon-coating. Replicas were examined under a JEM 100B Transmission electron microscope. Since original electron micrographs have black shadows, they are considered positive. Contact negatives of the original were made on Kodak medium contrast projector slide plates. These negatives were then used to make final prints with black shadow as observed in nature.

RESULTS

Cold Shock Hemolysis as a Function of Cooling Temperature and the Effect of High Osmolality

Intact cells, or the cells incorporated with 12-doxyl stearic acid, were suspended in 1,800 mOsm/kg NaCl solutions at 30° for 5 min, and then cooled to −5° at the rate of 9°C/min. Hemolysis during cooling was monitored by light scattering. Hemolysis began when the temperature dropped below ~10°, increased as the temperature went lower, and was complete at −5°C. There was no significant difference in the amount of hemolysis and the onset temperature of hemolysis between the cells with and without spin probes (Fig. 1).

Fig. 1 (inset) shows the effect of osmolality on cold shock hemolysis in the cells with and without spin probes. The cells were suspended in NaCl or sucrose solutions of various concentrations for 5 min at 24°, followed by cooling down to 0° in an ice bath at the rate of 60°C/min, and the degree of hemolysis was measured by hemoglobin quantitation. In NaCl solutions, hemolysis started when the osmolality exceeded 1,400 mOsm/kg, reached a maximum at 1,800 mOsm/kg, and then started decreasing above 2,200 mOsm/kg.

In hypertonic sucrose solutions, the onset of cold shock is seen at 1,200 mOsm/kg, but the maximum was reached at the same osmolality as in NaCl solutions. Again, there was no obvious difference either in the amount or the onset temperature of hemolysis between the intact cells and those in which the probes have been incorporated.

Cell Volume Change in Hypertonic NaCl Solutions

The cell volume in hypertonic solutions was determined by the standard hematocrit method. In hypertonic NaCl

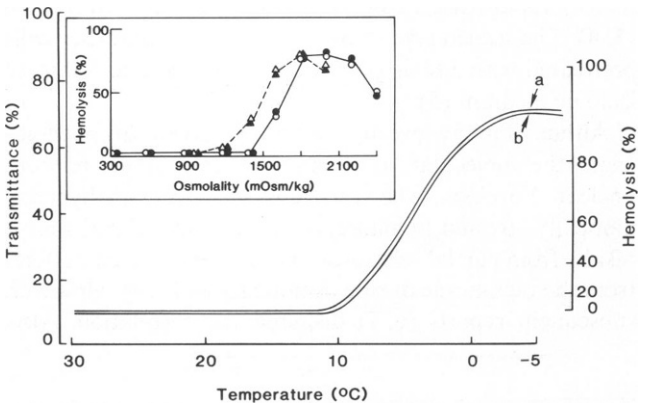

FIGURE 1 Cold shock hemolysis as a function of temperature during cooling measured as the increase in absorbance. (*a*) intact cells, (*b*) cells incorporated with a 12-doxyl stearate probe. *Inset:* cold shock hemolysis vs. osmolality (○, ●) in NaCl solutions, (△, ▲) in sucrose solutions. Open symbols show intact cells and closed symbols are for cells with 12-doxyl stearate probe.

solutions, the cell volume decreased steadily with increasing osmolality up to 900 mOsm/kg, at which point the volumme became ~60% of the original cell volume in 300 mOsm/kg NaCl solution (Fig. 2). The shrinking continued with a somewhat slower rate up to 1,400 mOsm/kg, and then the volume apparently started increasing above 1,400 mOsm/kg. The packing volumes in osmotically stressed cells have been measured in this laboratory using the ^{131}I-albumin method (15), and is known to show little change by hyperosmolality up to 1,200 mOsm/kg. Also, no cell-cell aggregation was detected in this condition under an optical microscope. Therefore, the apparent increase in volume most likely reflects a change in membrane permeability under centrifugal shear. The coincidence of the start of cold shock hemolysis and of the apparent cell volume increase both occurring at ~1,400 mOsm/kg is noteworthy.

Freeze-fracture Electron Microscopy

The condition of the hydrophobic interior of the membrane undergoing cold shock hemolysis was observed by freeze-fracture electron microscopy. Fig. 3 shows the appropriate features of the cell membrane treated at 24°, 10°, and 0°C in isotonic (plate *A*) or hypertonic (plates *B–F*) NaCl solutions. No change in the surface condition was observed in cells suspended in isotonic solutions even at 0°, whereas several marked wrinkles were clearly visible in osmotically stressed cells at 10° (plate *E*) and more clearly at 0°C (plate *F*). Under a larger magnification, the membrane particles appeared somewhat more aggregated, creating high density regions in hypertonic solutions (plates *C* and *D*) but not in isotonic condition (plate *A*).

Effect of Osmolality on Membrane Fluidity

Changes of cell membrane fluidity at varying osmolalities were measured at 24°C, using spin probes. The separation between the outer hyperfine extrema, $2A_\parallel$ was used as an indicator of the membrane fluidity, and its change with osmolality is shown in Fig. 4 *A*. No spectral change was observed by increasing osmolality when observed with 5-doxyl stearic acid, which probes near the polar surface of the bilayer. However, a steady increase in $2A_\parallel$ was seen up to 900 mOsm/kg when 12-doxyl and 16-doxyl stearate probes were used, which probe more toward the hydrophobic middle region of the bilayer. The fluidity change shows a break point near the osmolality, where the cell volume also reached 60%, suggesting a close connection between the fluidity decrease and the decrease in the cell volume due to hypertonic stress.

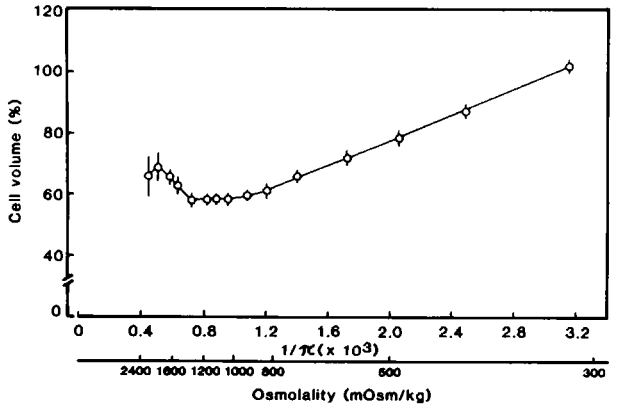

FIGURE 2 Cell volume change in hypertonic NaCl solutions. Relative volume indicates the ratio of hematocrit in isotonic PBS to that in NaCl solutions. π is osmolality. Each symbol and bar show the mean value ±SD (n = 6).

In EPR spectra of the cells incorporated with a probe of the cholesterol analog androstane, the overall spectral intensity showed a sharp decrease over the range of 1,200–1,500 mOsm/kg, as the salt concentration was increased at room temperature, with virtually no other change in spectral shape (Fig. 4 *B*). The intensity was estimated by the peak-to-trough height of the middle EPR line, since no systematic change in peak height ratios was detected. The cause of this partial loss of intensity is not completely clear (see Discussion).

In hypertonic sucrose solutions, 12-doxyl stearate showed a different feature in EPR spectra: as the sucrose concentration was increased, a new pair of absorptions emerged as shoulders on the outside of the original hyperfine extrema (Fig. 5). Due to the overlapping, precise determination of peak positions was not attempted in this case.

Effect of Cooling on Membrane Fluidity

The temperature dependence of membrane fluidity was measured in cell suspensions in isotonic and in 1,800 mOsm/kg NaCl solutions using the 12-doxyl stearic acid probe. The relation between $2A_\parallel$ and the temperature is presented as an Arrhenius-like plot of the rotational correlation time τ of the spin probe as shown in Fig. 6. The τ values were estimated from $2A_\parallel$ values using the equation that is approximately applicable to the present case (16), where, judging from the spectral shape, the rotational motion of the probe is in the intermediate region.

$$\tau = 8.52 \times 10^{-10} (1 - s)^{-1.16},$$

where $s = A_\parallel / A_z$ and A_z is the single crystal $A_\parallel$ values, whch is assumed to be 33.6 gs (17).

The plot for the control suspension shows two straight lines with a break at 10°, while that of the hypertonic suspension exhibits a clear discontinuity in $\tau \sim 10$°C.

DISCUSSION

There are at least four critical factors for inducing cold shock hemolysis: (*a*) the osmolality of the suspending medium, (*b*) cooling the suspension below 10°C, (*c*) incubation period and temperature in hypertonic suspension before cooling, and (*d*) the cooling rate (11). In the present article, the effects of the first two factors were studied by

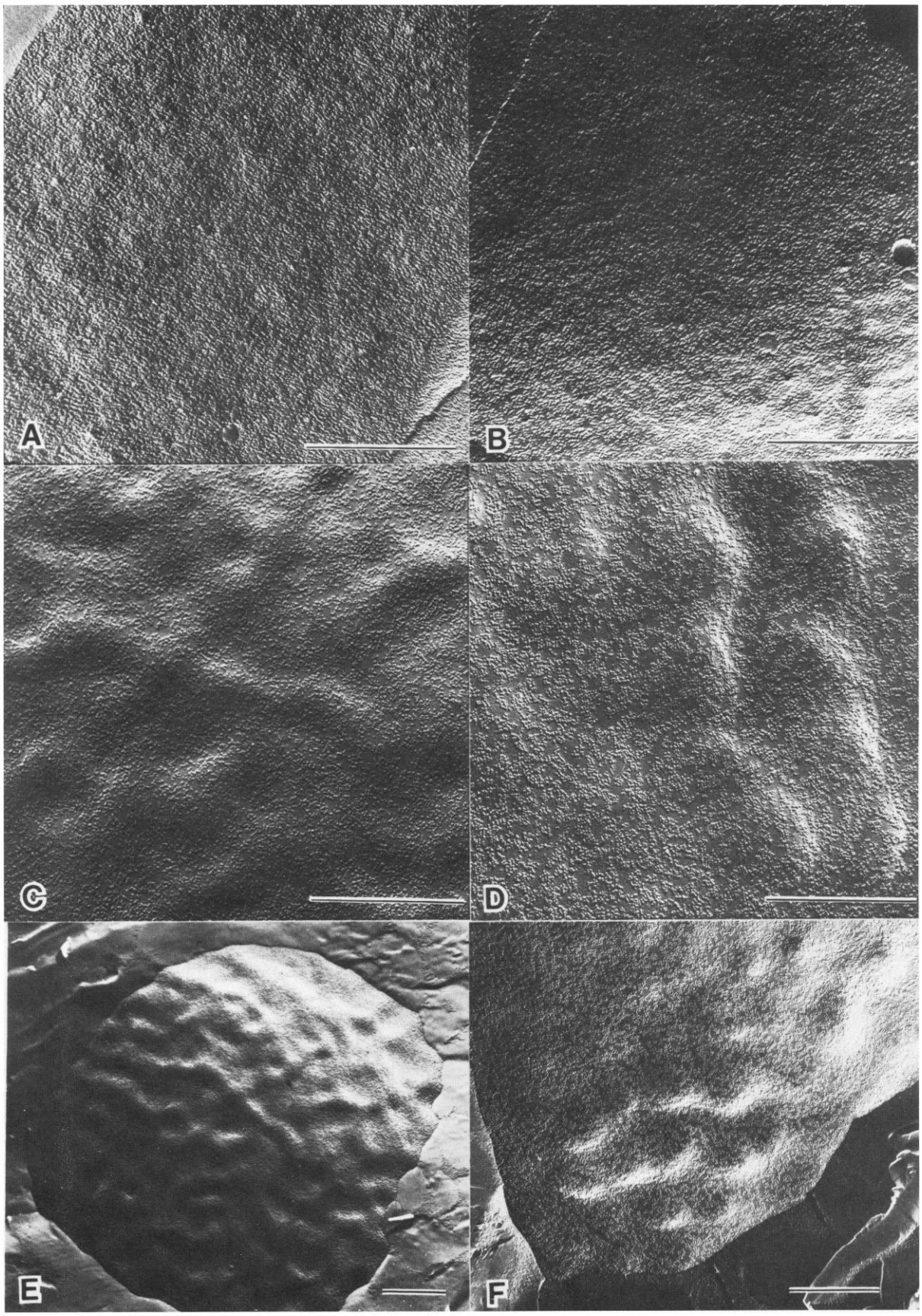

FIGURE 3 Freeze-fracture electron microscopy patterns. *P* faces of cells in isotonic solutions and of osmotically stressed cells at 24, 10, and 0°C *Plate A:* in isotonic PBS at 0°C; *Plates B–F:* in hypertonic NaCl solution (1,800 mOsm/kg). *B* at 24°, *C* and *E* at 10°, *D* and *F* at 0°C. Magnification: *A–D* × 55,000, *E* × 20,000, *F* × 29,000. The bars indicate 0.5 μm.

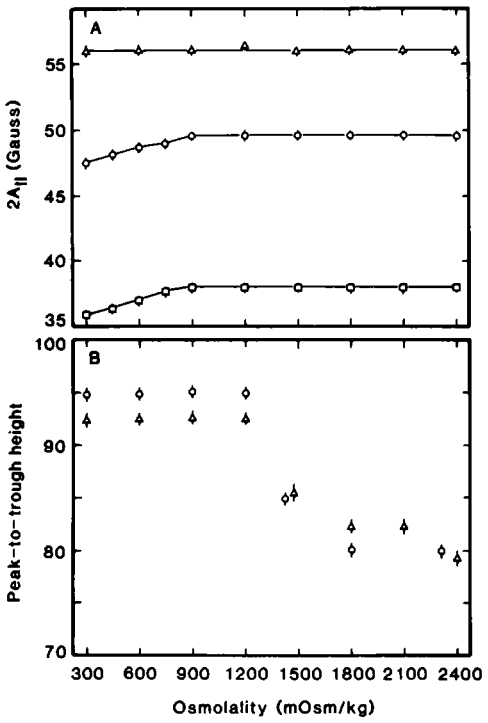

FIGURE 4 (*A*) Effect of osmolality on $2A_{||}$ of stearate probes in erythrocyte membrane suspended in NaCl solutions. (△) 5-doxyl, (○) 12-doxyl, (□) 16-doxyl stearate. (*B*) Peak-to-trough height of middle EPR line of androstane probe vs. osmolality. Results of two independent runs. Temperature was held constant at 24°C.

the spin probe EPR and freeze-fracture electron microscopy.

The spin probes, 5-, 12-, and 16-doxyl stearic acid, are used to probe localized areas in the membrane ~3, 10, 14 carbon-carbon bond lengths, respectively, from the polar

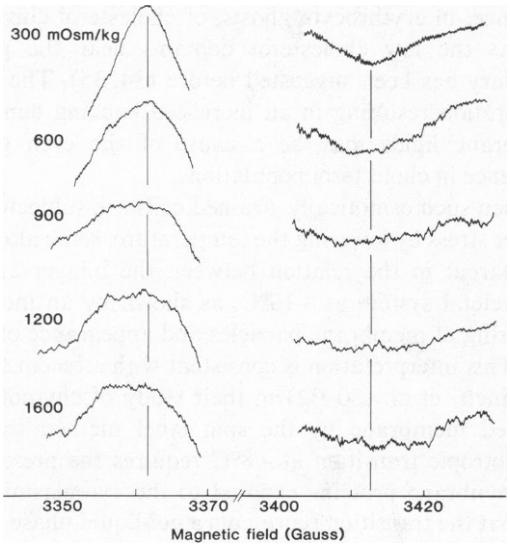

FIGURE 5 Appearance of strongly immobilized EPR components of 12-doxyl stearate in cells suspended in sucrose solutions. Only the outer hyperfine extrema are shown. The bars indicate the original peak positions in PBS.

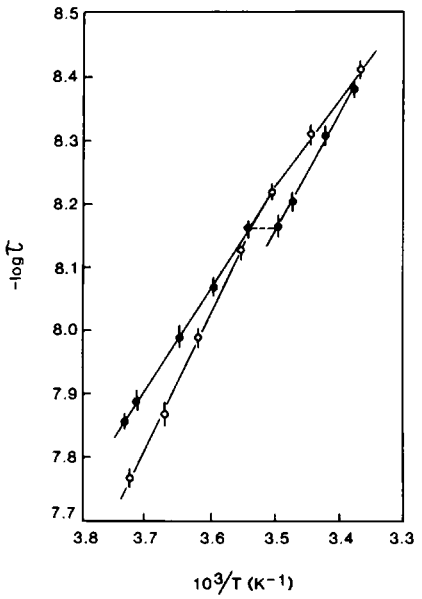

FIGURE 6 Rotational correlation time (τ) of 12-doxyl stearate vs. $1/T$. (○): in isotonic PBS, (●): in 1,800 mOsm/kg NaCl solution.

carboxylate group that is anchored at the membrane surface. The androstane probe, on the other hand, is considered useful for mimicking the motion of cholesterol in the membrane. In view of the absence of any significant difference in the cold shock characteristics due to the presence of spin probes (Fig. 1), the information obtained by the EPR method may be assumed to be valid for the actual events occurring in the cell.

Since both hypertonic NaCl and sucrose solutions induce cold shock hemolysis, the elevated osmolality, as opposed to the electrolyte concentration, plays an important role in inducing cold shock. While the end result is the same in the two solutions, they also display several noteworthy differences: during prolonged exposure to hypertonic NaCl solution, the extent of subsequent cold shock hemolysis is reduced, whereas the prolonged exposure to sucrose increases hemolysis (11). There is also a significant difference in EPR spectra in cells exposed to the two solutions (Fig. 5), indicating that sucrose solutions induce the strongly immobilized, along with the less immobilized regions in the membrane. This may be related to the observed onset of hemolysis at a lower osmolality, 1,200 mOsm/kg, compared with 1,400 mOsm/kg in NaCl solution.

In the previous report, we have emphasized the importance of partial loss in cell volume as a sensitizing process for hemolysis (11). The results presented here show that the membrane fluidity in the inner region of the bilayer decreased as the suspension osmolality was increased up to 900 mOsm/kg, above which level no further change was detected by stearic acid spin probes. During this process, the cell volume decreased to ~60% of the original size. That there is some further change in the membrane state around 1,200 mOsm/kg was demonstrated by the andro-

stane probe by sudden decrease in the spectral intensity. The most plausible explanation of the partial loss in intensity is that androstane, and possibly also cholesterols, in the membrane form loose aggregations, and that the spectral lines are suppressed due to dipolar broadening. Extrusion of the probe from the cell into the extracellular medium to form vesicles can be excluded, but release of the probe into the inside of the cell to be eventually reduced by cytosolic components (18) cannot be completely ruled out.

The second determinant of cold shock, cooling below 10°C, was studied using 12-doxyl stearate in the cells suspended in 1,800 mOsm/kg NaCl solution. The Arrhenius-like plot of the rotational correlation time for the control consists of two lines with a slight break at around 10°C, which is not associated with cold shock (Fig. 6). In contrast, the plot in the hypertonic state showed a stepwise shift around the same temperature, as if there is some type of a phase transition. Although details of the underlying mechanism of the abrupt change are not completely understood, it seems evident that some drastic change in the probe motion has occurred around this temperature. The straightforward interpretation of the regression lines may be that at temperatures above this critical change, the slope of the line is greater in the osmotically stressed system than in the control, implying that the activation energy of the probe motion is higher under the osmotic stress than in the control. The shift of the line around the critical point would indicate that the same degree of probe motions that existed before the event can be sustained by a lower temperature after the change has taken place in the membrane. Below the critical temperature, the relation between the stressed system and the control, with regard to the activation energy, is reversed, implying partial relief of the stress in the cells in hypertonic solution.

While the above explanation of the state of the probe motion should be based rigorously on results of spectral simulation, it is supported by the observations obtained by freeze-fracture electron microscopy that presented intramembranous morphological change as a function of cooling (Fig. 3). In addition to the appearance of aggregation of membrane particles in osmotically stressed cells at ~10°C, there emerged clearly visible wrinkles covering the whole inner boundary surface, an expression of strain in the membrane. The wrinkles appeared to grow into bumps as the temperature was lowered to 0°C. Judging from the size, it is unlikely that the wrinkles reflect the ice crystal formation inside the cell.

Under an optical microscope, the morphological change at ~10°C in hypertonic solution was observed as a rapid transition from flattened discoid shapes to spherocytes (19). Such drastic change of cell shape and wrinkling in inner layer surface suggest alteration of the connection between the cytoskeletal network and the membrane bilayer (20). It must be noted that these morphological changes observed at 10°C are the features existing before, and not the results of, hemolysis, which at this temperature is barely starting. Also, aggregations of membrane particles in ghosts under various conditions are well-documented (21), but aggregations before hemolysis, as observed here, are rather uncommon.

The meaning of the inflection points, observed in various physico-chemical measurements of the membrane properties as a function of temperature, in terms of the actual change in the membrane state or structure have been the subject of numerous studies (22–32). Use of different probes and techniques yielded assignments of somewhat different temperatures for such thermotropically induced transitions, and the exact nature of them is variously interpreted, but it is generally agreed now that the inflections observed in the medium temperature range approximately between 13° and 20°C are real, and reflect a discrete change of fluidity in membrane bilayer including the lipid-protein boundary.

On the other hand, the inflections observed, in the present work, in the $2A_{\parallel}$-osmolality curves (Fig. 4 *A*) are interpreted not to represent such a transition, but implies a steady increase in order in the membrane due to the osmotic stress up to a certain limit imposed by the membrane structure. This, then, is followed by what appears to be a transition occurring as the osmolality is further increased, which indicates the creation of domains having, respectively, a higher and a lower cholesterol population (Fig. 4 *B*). The low cholesterol domains can, in principle, be more susceptible to a thermotropic phase transition as shown by the x-ray diffraction studies of extracted membrane lipids (33). Clearly, the nature of these osmotically induced consequences should be different from those affected by only temperature change described in the past as phase transitions (e.g., 27, 29, 30), because merely lowering the temperature does not lead to a hemolysis. The existence, in erythrocyte ghosts, of cholesterol clusters as well as the low cholesterol domains near the protein boundary has been suggested before (34, 35). The severe dehydration resulting in an increased packing density of membrane lipids may be a cause of the even greater difference in cholesterol populations.

When such osmotically strained cells are subjected to a further stress by lowering the temperature, some alteration is apparent in the relation between the bilayer and the cytoskeletal system at ~10°C, as shown by an increased clustering of membrane particles and appearance of wrinkles. This interpretation is consistent with a recent finding by Minetti et al. (30–32) in their study of chymotrypsin digested membrane by the spin label method that the thermotropic transition at ~8°C requires the presence of the membrane proteins exposed to the cytoplasmic side, and that the transition represents a gel-liquid phase transition (30). Also, the increased particle clustering, reducing the lipid-protein boundary area, may lead to an overall instability of the membrane system. All these changes

would prepare for the hemolysis that follows. The actual site of lesion, however, could not be definitely determined in this work, since only a few slits in the membrane, transiently opened (36), may be sufficient to cause a hemolysis. The low cholesterol region bounded by clusters of membrane particles, undergoing a transition at the critical temperature, may be a probable candidate for the site of lesion.

In summary, we have presented here experimental evidence for the stress in the erythrocyte membrane created by hyperosmolality. The membrane thus stressed seems to undergo a drastic reorganization at ~10°C. The appearance of wrinkles and budding in membranes in that temperature range supports the view that the reorganization in the state of the membrane system including the cytoskeleton may play a key role in the phenomenon of cold shock hemolysis.

The authors would like to thank Drs. H.T. Meryman and R.J. Williams for their valuable suggestions and technical help.

Received for publication 6 May 1985 and in final form 4 October 1985.

REFERENCES

1. Farrell, J., and A. H. Rose. 1967. Temperature effects on microorganisms. *In* Thermobiology. A. H. Rose, editor. Academic Press Inc., Ltd., London. 142–169.
2. Morris, G. J., and P. F. Watson. 1984. Cold shock injury—a comprehensive bibliography. *Cryo Lett.* 5:352–372.
3. Lovelock, J. E. 1954. Physical instability and thermal shock in red cells. *Nature (Lond.).* 173:659–667.
4. Lovelock, J. E. 1955. Haemolysis by thermal shock. *Br. J. Haematol.* 1:117–129.
5. Möllby, R. 1978. Bacterial phospholipases. *In* Bacterial Toxins and Cell Membranes. J. Jeliaszewicz and T. Wadstrom, editors. Academic Press Inc., New York. 367–427.
6. Morris, G. J. 1975. Lipid loss and heamolysis by thermal shock: lack of correlation. *Cryobiology.* 10:119–125.
7. Green, F. A., and C. Y. Jung. 1977. Cold shock induced hemolysis in a hypertonic milieu. *J. Membr. Biol.* 33:249–262.
8. Dubbelman, T. M. A. R., A. W. de Bruijne, K. Christianse, and J. van Steveninck. 1979. Hypertonic cryohemolysis of human red blood cells. *J. Membr. Biol.* 50:225–240.
9. Green, F. A., C. Y. Jung, J. Cuppoletti, and N. Owens. 1981. Hypertonic cryohemolysis and the cytoskeletal system. *Biochim. Biophys. Acta.* 48:225–230.
10. Green, L. A. D., H. L. Hui, F. A. Green, C. Y. Jung, and W. S. Pudlak. 1983. The role of choline phospholipids in hypertonic cryohemolysis. *Cryobiology* 20:25–29.
11. Takahashi, T., and R. J. Williams. 1983. Thermal shock hemolysis in human red cells. I. The effect of temperature, time and osmotic stress. *Cryobiology.* 20:507–520.
12. Nunes, M.d'A. 1982. A spin label study of erythrocyte membrane during simulation of freezing. *J. Membr. Biol.* 60:155–162.
13. Roussellet, A., G. Claudine, J. Matricon, A. Bienvenue, and P. F. Devaux. 1976. Study of the transverse diffusion of spin labeled phospholipids in biological membranes. I. Human red blood cells. *Biochim. Biophys. Acta.* 426:357–371.
14. Steere, R. L. 1981. Preparation of freeze-fracture, freeze-etch, freeze-dry and frozen surface replica specimens for electron microscopy in Denton DFE-2 and DFE-3 freeze-etch units. *In* Current Trends in Morphological Techniques. Vol. 2. J.E. Johson, Jr., editor. CRC Press Inc., Boca Raton, FL. 131–181.
15. Meryman, H. T. 1971. Osmotic stress as a mechanism of freezing injury. *Cryobiology.* 8:489–500.
16. Freed, J. H. 1976. Theory of slow tumbling ESR spectra for nitroxides. *In* Spin Labeling. L. J. Berliner, editor. Academic Press, Inc., New York. 53–132.
17. Gaffney, B. J. 1976. Practical considerations for the calculation of order parameters for fatty acid or phospholipid spin labels in membranes. *In* Spin Labeling. L. J. Berliner, editor. Academic Press Inc., New York. 567–571.
18. Seigneuret, M., and P. F. Devaux. 1984. ATP-dependent asymmetric distribution of spin-labeled phospholipids in the erythrocyte membrane: relation to shape changes. *Proc. Natl. Acad. Sci. USA.* 81:3751–3755.
19. Williams, R. J., and T. Takahashi. 1984. Erythrocyte metastability: microscopic observation of thermal shock hemolysis. *Cryo Lett.* 5:111–116.
20. Haest, C. W. M. 1982. Interactions between membrane skeleton proteins and the intrinsic domain of the erythrocyte membrane. *Biochim. Biophys. Acta.* 694:331–352.
21. Pinto da Silva, P. 1972. Translational mobility of the membrane intercalated particles of human erythrocyte ghost. *J. Cell Biol.* 53:777–787.
22. Zimmer, G., H. Schirmer, and P. Bastian. 1975. Lipid-protein interactions at the erythrocyte membrane. Different influence of glucose and sorbose on membrane lipid transition. *Biochim. Biophys. Acta.* 401:244–255.
23. Tanaka, K., and S. Ohnishi. 1976. Heterogeneity in the fluidity of intact erythrocyte membrane and its homogenization upon hemolysis. *Biochim. Biophys. Acta.* 426:218–231.
24. Bieri, V. G., and D. F. H. Wallach. 1976. Lipid-protein relationships in erythrocyte membranes revealed by paramagnetic quenching of protein fluorescence. *Biochim. Biophys. Acta.* 443:198–205.
25. Weltzien, H. U., B. Arnold, and H. G. Kalkoff. 1976. Quantitative studies on lysolecithin mediated hemolysis. Benzylated lysolecithin as a probe to study effects of temperature and red cell species on the hemolytic reaction. *Biochim. Biophys. Acta.* 455:56–65.
26. Galla, H.-J., and J. Luisetti. 1980. Lateral and transversal diffusion and phase transitions in erythrocyte membranes. An excimer fluorescence study. *Biochim. Biophys. Acta.* 596:108–117.
27. Ogiso, T., M. Iwaki, and K. Mori. 1981. Fuidity of human erythrocyte membrane and effect of chlorpromazine on fluidity and phase separation of membrane. *Biochim. Biophys. Acta.* 649:325–335.
28. Janoff, A. S., D. L. Mazorow, R. T. Coughlin, A. J. Bowdler, A. Haug, and E. J. McGroarty. 1981. The modification of human erythrocyte membrane structure by membrane stabilizers: an electron spin resonance study. *Am. J. Hematol.* 10:171–179.
29. Herrmann, A., K. Arnold, G. Lassman, and R. Glaser. 1982. Structural transitions of erythrocyte membrane: an ESR approach. *Acta Biol. Med. Ger.* 41:289–298.
30. Minetti, M., and M. Ceccarini. 1982. Protein dependent lipid lateral phase seperation as a mechanism of human erythrocyte ghost resealing. *J. Cell. Biochem.* 19:59–75.
31. Minetti, M., M. Ceccarini, and A. M. M. Di Stasi. 1984. Role of membrane thermotropic properties on hypotonic hemolysis and hepertonic cryohemolysis of human red blood cells. *J. Cell. Biochem.* 25:61–72.
32. Minetti, M., M. Ceccarini, and A. M. M. Di Stasi. 1984. Characterization of thermotropic structural transition of erythrocyte membrane: A biochemical and electron paramagnetic resonance approach. *J. Cell. Biochem.* 25:73–86.
33. Gottlieb, M. H., and E. D. Eanes. 1974. On phase transitions in erythrocyte membranes and extracted membrane lipids. *Biochim. Biophys. Acta.* 373:519–522.
34. Higgins, J. A., N. T. Florendo, and R. J. Burrnett. 1973. Localization

of cholesterol in membranes of erythrocyte ghost. *J. Ultrastruct. Res.* 42:66–81.

35. Bieri, V. G., and D. F. H. Wallach. 1975. Variations of lipid-protein interactions in erythrocyte ghost as a function of temperature and pH in physiological and non-physiological ranges. A study using paramagnetic quenching of protein fluorescence by nitroxide lipid analogues. *Biochim. Biophys. Acta.* 406:415–423.
36. Lieber, M. R., Y. Lange, R. S. Weinstein, and T. L. Steck. 1984. Interaction of chlorpromazine with the human erythrocyte membrane. *J. Biol. Chem.* 259:9225–9234.