Kawasaki Med. J. Vol. 2: No. 4, 199-209, 1976

WARTY ERYTHROCYTE GHOST PRODUCED BY RECTILINEARLY INCREASING HYPOTONIC STRESS IN COIL PLANET CENTRIFUGATION

Osamu YAMADA

Division of Hematology
Department of Medicine, Kawasaki Medical School,
Kurashiki, Okayama, 701-01, Japan

Accepted for Publication on Jul. 21, 1976

Abstract

Coil planet centrifugation provides an excellent measure for observation of erythrocyte hemolysis under continuously increasing hypotonic stress which takes place in a long (3 m) slender (bore diameter 0.3 mm) coiled polyethylene tube filled with a column of NaCl solution of rectilinearly decreasing osmolarity of 120 → 50 mOsM. From one end of the coiled tube a minute amount (10 μl) of blood sample was introduced, tightly-sealed at both ends, and then subjected to coil plant contrifugation. After centrifugation the coiled tube containing hemolysis band was divided into 15 segments of equal length. The contents of the segments were discharged from the tube separately so that erythrocyte stromas in the hemolysis band might be fixed in glutaraldehyde and osmic acid for stereoscan electron microscopy.

The result of the observation disclosed a considerably large number of stromas engraved with tortoise-shell like irregular mosaic pattern, which reminded us of Bull's theory of mosaic structure of erythrocyte membrane. However, the mosaic pattern was composed of numerous warts which were too large in size to be regarded as individual units constructing the erythrocyte membrane. It is therefore presumed that the warty appearance of the stroma will be the shrinkage-product of erythrocyte ghost possessing a tortoise-shell like contractile framework inside of the cellular membrane after the erythrocyte has discharged its content by hypotonic stress.

INTRODUCTION

A minute amount (10 μl) of coagulation-prevented blood sample obtained from normal subjects is put into a coil of a slender polyethylene tube (bore diameter, 0.3 mm; length, 3 m) tightly entwinded about an acrylite rod (length, approximately 20 cm) which is filled with a column

of salt solution giving a rectilinearly decreasing osmotic gradient from 120 to 50 mOsM, and fuse-sealed by heating at both ends in order to employ it as an equivalent of a centrifuge tube.

If this "coil" is inserted into the coil-holder of a coil planet centrifuge and centrifugation is started, the erythrocytes of the blood sample will migrate in the lumen of the "coil" from higher osmolarity end (120 mOsM) to the lower osmolarity side at a constant rate (0.5 cm/sec) by centrifugal force. In the course of this migration, erythrocytes leak their contents gradually through the membranes due to ever-increasing hypotonic stress and entirely hemolysed individually at the site where their membranes can no longer withstand the hypotonicity, producing en masse a hemolysis band in the coil. In the previous communication it was pointed out that the contents of the erythrocytes leaked outside at a speed inversely proportional to the order of their molecular weights[1], and it was also suggested that the mechanism of formation of the hemolysis band in the coil might be revealed by the electron microscopic examination of the erythrocyte ghost (or stroma) obtainable from the "coil", although optical microscopy was not helpful. Accordingly, ghosts present in the hemolysis band were collected, fixed, evaporated in vacuum to be scrutinized by an electron scan microscope.

Unfortunately, the hemolysis mechanism was not successfully elucidated by this experiment. However, a great number of erythrocyte ghosts with a peculiarly strange morphology which had never been described happened to be visualized. They looked like deformed discs with tortoise-shell mosaic appearance. Under high magnification it was seen that they produced warts all over the surface. Of course, ghosts with smooth surface were encountered along with them. However, the incidence of warty ghosts (ratio to the total number of ghosts) and the hemoglobin concentration curve of the hemolysis band varied closely in quite a good accordance. Therefore, it was supposed that the warty ghosts would be the end product of erythrocyte hemolysis. This paper aims to present the result of our observation together with the photographic pictures of the warty ghosts.

MATERIALS AND METHODS

Blood samples (about 1 ml) were collected into an aseptic syringe from the antecubital vein of normal subjects transferred to a test tube containing Anticlot-Et (1 drop), and mixed by agitation to prevent coagulation. An aliquot (10 μl) of blood thus obtained was introduced

into a coil (possessing NaCl solution column with rectilinearly decreasing osmolarity of 120 → 50 mOsM), fuse-sealed by heating at both ends to submit it to coil planet centrifugation (revolution, 1600 RPM; selfrotation, 16 RPM; for 10 min.) in the same manner as described in the previous communication.[1,2]

The polyethylene tube containing a hemolysis band was unwound from its acrylite rod, and cut into 15 segments of equal length. A portion of the content of each segment was transfused into a cup individually for the determination of hemoglobin concentration, while the remaining portion (about 100 μl) was blown out of the segment over a fragment (7×7 mm) of a cover glass to sediment the ghosts contained in it.

(1) Hemoglobin concentration.

Hemokit-N color reagent (Nippon-shoji) was diluted 50 times with H_2O. Aliquot of 10 μl of segment content was added to 2.5 ml of the diluted color reagent with an ultramicropipet in order to convert hemoglobin into cyan-methemoglobin, the optical density of the colored solution was measured at 420 nm (Soret band)[2], in a spectrophotometer. The hemoglobin concentration was read by collation of the optical density with the calibration curve.

(2) Electron scan microscopy of ghosts.

The ghosts contained in the solution of the segment placed on the cover glass (7×7 mm) mentioned above were left standing and sedimented freely for 10 minutes. Subsequently the cover glass was soaked in glutaraldehyde solution (1 %) for 1.5 hours for fixation of the ghosts, transferred into phosphate buffer solution (0.1 M, pH 7.2) for washing for 1 hour, and then into osmic acid solution ($OsO_4 : H_2O = 1 : 100$) for refixing (1 hour). It was washed again with Milloring's buffer solution[3] and dehydrated with ethanol of ascending concentrations (25 → 100 %). This dehydrated cover-glass specimen was immersed in ethanol-isoamyl acetate mixture (1 : 1) for 5 min., transferred into isoamyl acetate solution (100 %) for 10 min., subjected to critical point drying (Hitachi HCP-1) and coated with gold-palladium in an Eiko IB-2 ion cleaner. The cover-glass specimen thus prepared was used for electron scan microscopy.

In electron scan microscopy (Hitachi HHS-2) the distributions of erythrocytes and ghosts were examined at 50-3,000 diameters and the surface of their membranes at 10,000-20,000 diameters.

RESULTS

Figere 1 is the illustrative summary of the various erythrocyte ghosts seen electron scan microscopically in the fluid of hemolysis band. These ghosts can be divided into two groups. One is the group having

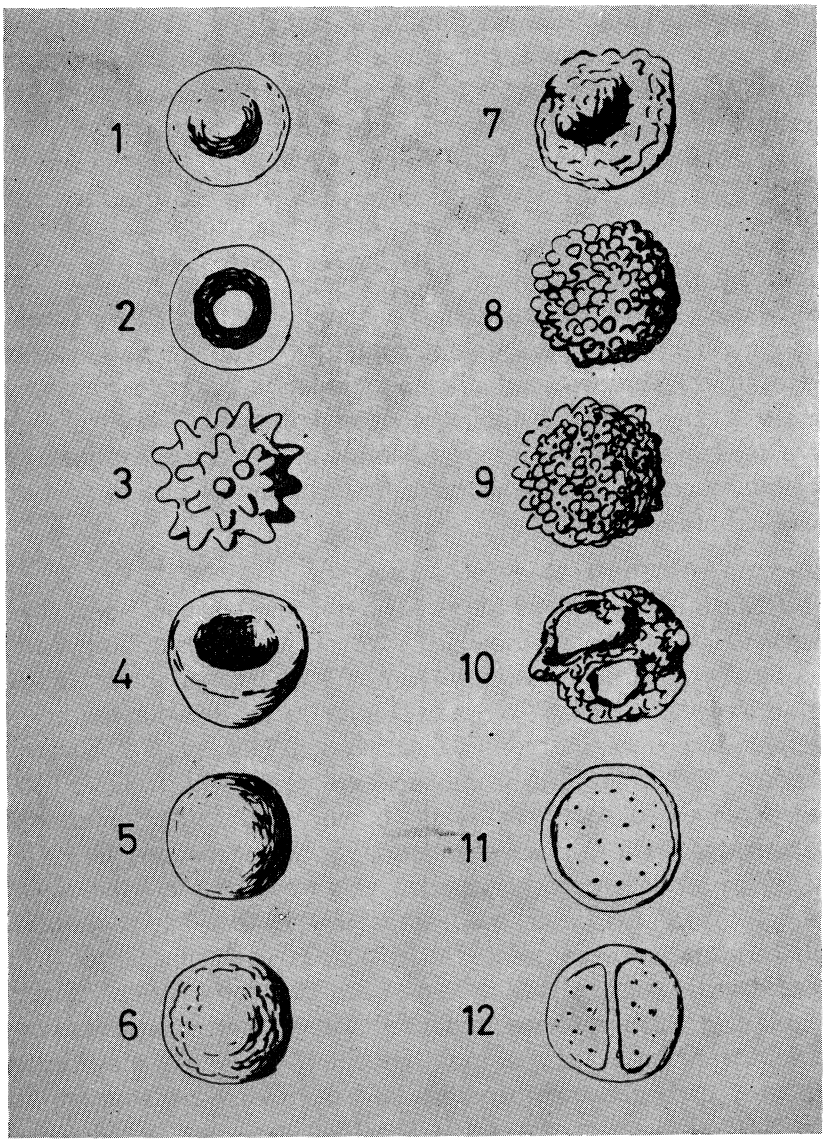

Fig. 1. Schematic illustration of the classification of ghosts as observed by electron scan microscopy.

smooth surface (1-5) and another showing uneven and strange-looking surface (6-12).

The morphologically strange-looking ghosts are characterized by the cellular membrane with tortoise-shell network crowded with warts as shown by the cells 8-9 illustrated in Figure 1. The warts vary in size, ranging from 0.05 to 0.2 μm in diameter.

The viscosity curve of the incidence of strange-looking ghosts relative to whole ghosts (%) run closely parallel to the curve of hemoglobin concentration over the whole range of hemolysis band with complete coincidence of their peaks and bases. This is clearly recognized by Figure 2.

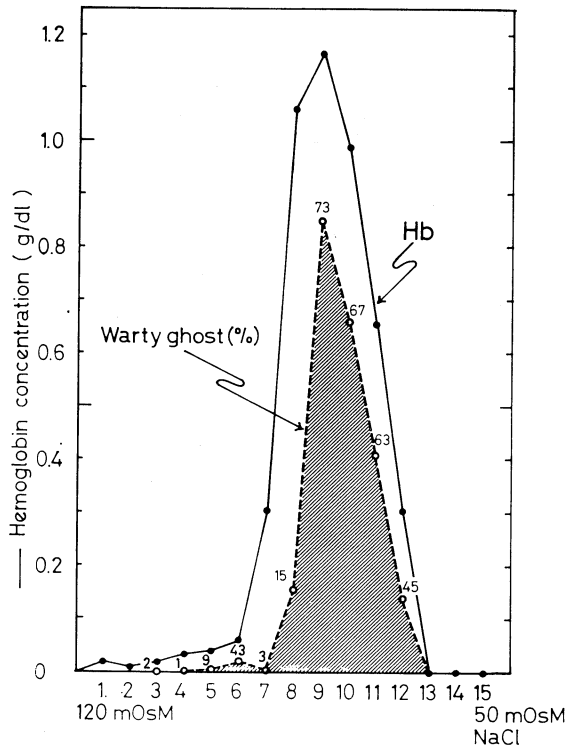

Fig. 2. Incidence of warty ghosts (expressed in percentage of the total ghost population; shaded area) in relation to the continuous decrease in osmolarity. Hemolysis curve is presented for the purpose of comparison.

DISCUSSION

When erythrocytes and ghosts in the fluid of the segments of the

coil were examined at low power magnification (950×) of electron scan microscope, it was noticed that the ghosts having released their contents were smaller than the perfect erythrocytes, looking like a thin ring as shown in Figure 3. Their warty surface was just brought to vision, when the cover-glass specimens were scrutinized at high power magnification of 20,000 ×. High-power magnification a typical warty ghost is presented in Figure 4. Ghosts of varied morphology are assembled in Figure 5.

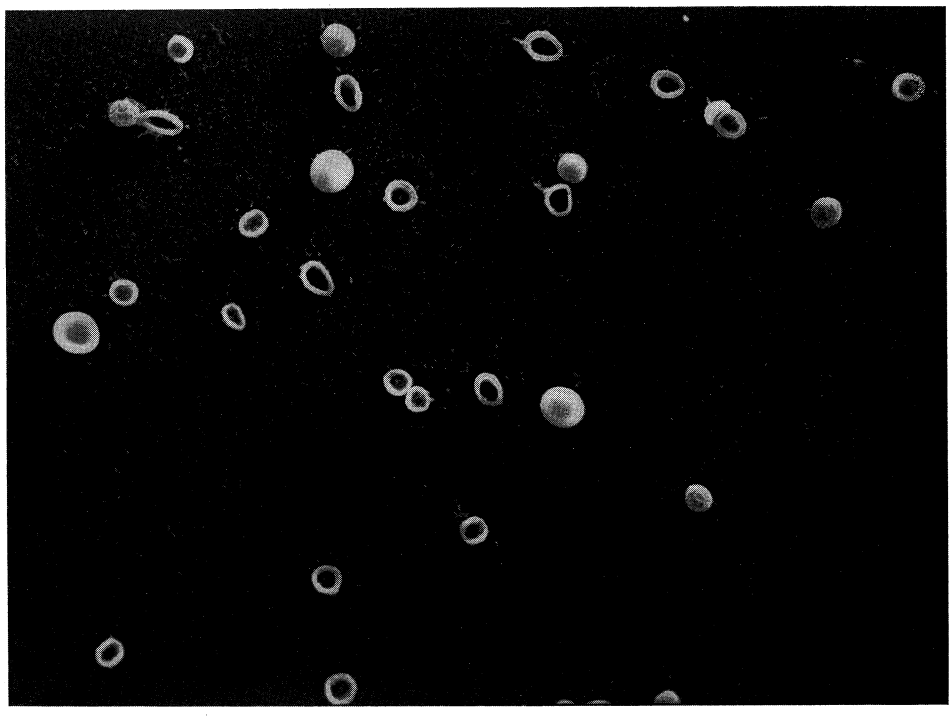

Fig. 3. Low-power magnification (950×) of ghosts by electron scan microscopy.

The ghosts with smooth surface are really the same in size as the untreated erythrocytes (Figure 1, 1-5, with only a few wrinkles on the membrane surface at the most) and those with warty membrane surfaces (Figure 1, 6-12) are significantly smaller, when they are compared by the measurement of their diameters (longitudinal and horizontal). This is clearly noticed from the lines representing the two sorts of the ghosts illustrated in Figure 6. It is, therefore, germane to interpret that the ghosts with warty membrane surface are the end product of those with

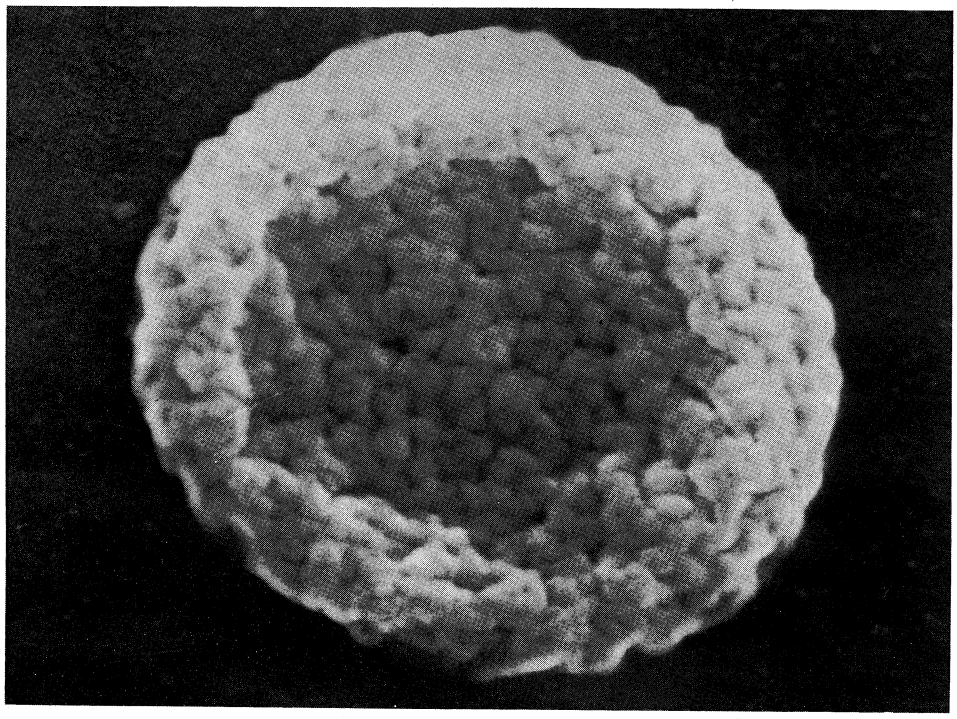

Fig. 4. High-power magnification (20,000×) of a typical warty ghost by electron scan microscopy

smooth surface by contraction and shrinkage after complete hemolytic discharge of the content.

From the pictures of ghosts presented in Fig. 4 and 5, bearing the phenomena mentioned above in mind, it is presumed that hemolysis of erythrocytes caused by hypotonic stress will proceed in the following steps.

Namely, erythrocytes have a cellular membrane with fluid mosaic structure of lipid double layers (inner and outer, consisting of cholesterol, phospholipid, etc.) randomly studded with varied protein column all over as propounded by Singer-Nicholson.[4] In addition, the membrane is coated inside with loosely meshed network of contractile protein (e. g. spectrin).[5,6] With the increase in hypotonic stress in the environment the erythrocyte membrane become increasingly permeable to various intraerythrocytic substances on account of loosening of its lipid double layer structure caused by swelling with water. Thus the substances including potassium, enzymes and hemoglobin, leak from inside of the

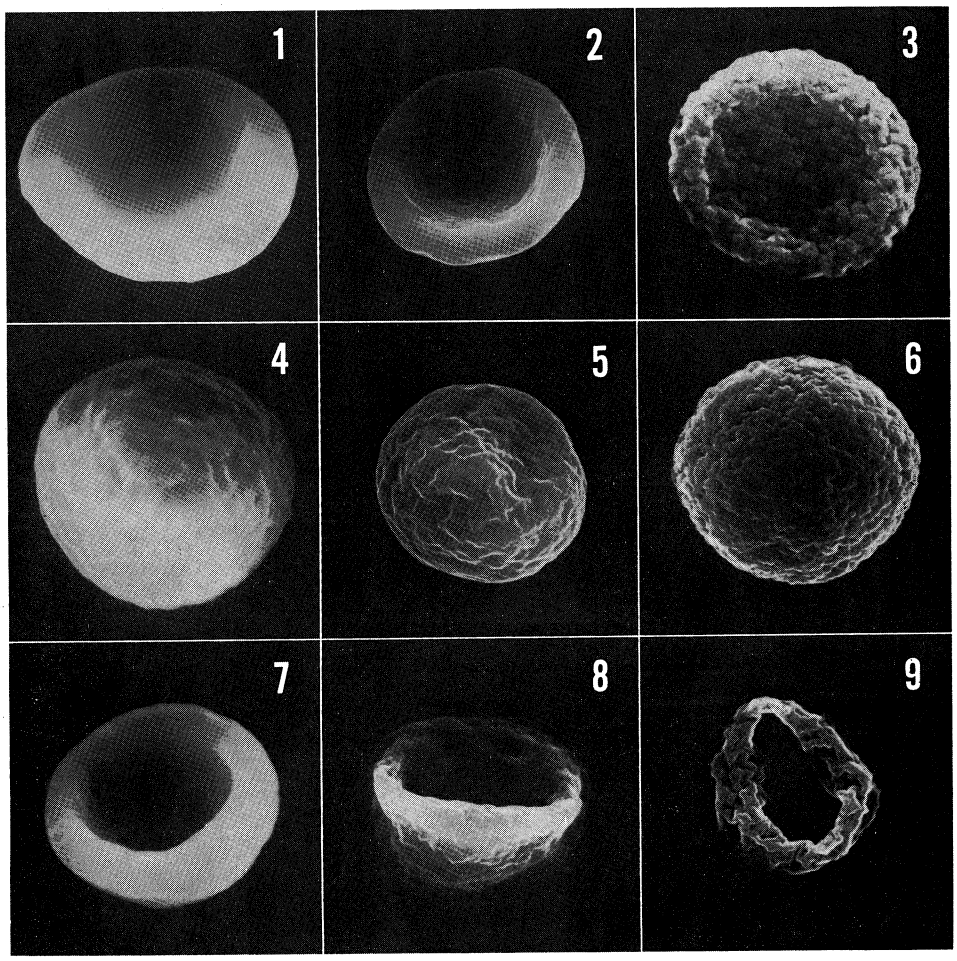

Fig. 5. Electron scan microscopic appearance of ghosts of varied morphology.
1, 3, and 2: discocytes. 4, 5, and 6: spheroid cells. 7, 8, and 9: stomatocytes.
1, 4, and 7: ghosts obtained on the high (around 120 mOsM) osmolar side.
2, 5, and 8: ghosts encountered in the intermediate zone of osmolarity (about 100 mOsM).
3, 6, and 9: ghosts seen in the fluid obtained on the low olmolar side (60–70 mOsM).

membrane to the outside milieu with the speed just inversely proportional to their molecular weights. Unfortunately, elucidation of the mechanism of hemolysis in its early stage was beyond the scope of electron scan microscopy, in our experiment. It has been said that discocytes become spherocytes through the stage of echinocytes by externalizafion of their membrane (exocytosis) producing projections out-

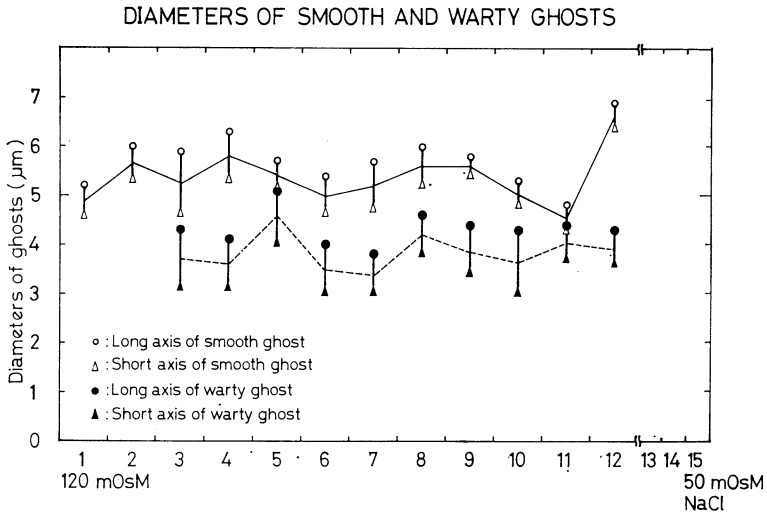

Fig. 6. Comparison of the size between the smooth ghosts (solid line) and the warty ghosts (broken line). On the abscissa the osmolarity of the fluids in the coils was taken for the sake of reference.

ward.[5] Conversely, when internalization of membrane is predominant, one side of the surface of discocyte becomes depressed and hollow, the erythrocytes are changed to assume the shape of a cup, i.e. stomatocytes.[5] Hemolysis in the hypotonic environment has been thought to take place after passing through these metamorphosis. What was brought to our vision by electron scan microscopy in our experiment was not only related to these metamorphoses, but also to the ghosts of wrinkled or uneven cellular membrane with profuse vegetation of warts. It is true that these findings would not be characteristic of dynamic observation of erythrocyte membrane proporties which employs a coil planet centrifugation, but they are seen also by static osmotic fragility tests of erythrocytes such as Parpart method.[7] However, in our experience coil planet centrifugation proved to be much superior to Parpart method for the purpose of quantitative analysis of erythrocyte ghosts.

According to Kawagoe et al.,[8] in coil planet centrifugation, the erythrocytes hemolyzed around the starting point of hemolysis band (higher osmolar side) belong to aged cells, while the erythrocyte hemolysed around the hemolysis end point (lower osmolar side) to younger cells (including reticulocytes). Perhaps, aged erythrocytes may have wasted plenty of substances (enzymes, proteins and lipids) required for

the maintenance of cellular integrity and have consumed the contractile network substances (such as spectrin) which coats the inner surface of their cellular membrane. Accordingly, the ghoast or stroma will no longer be able to contract after the relevant erythrocyte has extruded its content almost completely. That will be the reason why the ghosts collected from the portion of hemolysis starting point are without wrinkles on their surface membrane. They are unable to contract enough to produce wrinkles. On the contrary, the erythrocytes which are hemolysed in the middle portion of hemolysis band can diminish their volume significantly as their contents continue to leak out through the membrane, because they are young and possess rich contractile network substances inside the lipid double layers. While the contraction remains slight or mild, wrinkles or corrugation will appear over the surface of the membrane, but if the contraction proceeds to the extreme, the ghost will shrink until it produces a mixture of numerons warts and grooves over its surface. This is what is called warty ghost. The erythrocytes which have been rolled to the portion of hemolysis end point through the coil are very young. Therefore, their membrane composed of lipid double layers may be sufficiently strong and the membrane lipid composition may be adequate to keep the contents inside preventing them from leakage through the membrane (as for instance, membrane having plenty of cholesterol). Beside, even if the contents may have leaked out, the membrane will be so tough as to resist the shrinkage exerted by the contractile network of the inner coat of the membrane. So, it is thought that the ghosts retain their original smooth membrane or restore easily its original shape and volumn fast after extrusion of their contents to the external environment.

If the account described above is correct, it is conceivable why the incidence curve of the strange-looking ghosts rum parallel with the hemogblobin concentration curve and why the starting points, peaks and end points of both of these curves come to coincide. The strange-looking ghosts with profuse vegetation of warts on their membrane suggests therefore, that its original erythrocytes were "sound" and normal, while the ghosts with smooth surface will be considered to be derived either from aged erythrocytes having wasted contracted substances or from erythrocytes of which lipid double layers if sufficiently tough to overcome the strength of their contractile network (namely, reticulocytes or erythrocytes with cellular membrane of high cholesterol contents).

The outward appearanee of ghosts crowded with warts all over their membrane surface may remaind us of the hypothesis propounded by Bull[9] that the erythrocyte membrane has tortoise-shell frame work. However, in reality, the warts actually seen on the surface are too large and too much varied in size to warrant them as the minimum unit of cellular membrane. It should accordingly be considered that the strange-looking warty ghosts are the hemolysis end product brought about by shrinkage of the contractile framework lying underneath the fluid lipid double layers of the membrane.

Grateful acknowledgements are due to Professor Susumu Shibata for the kind advice for preparation of the manuscript, and to Mr. Kosaku Goto, Miss Takako Ando, Miss Mizue Kubotsu for technical assistance.

This investigation was supported in part by the grant from the Kawasaki Medical School (Research Project 49202).

REFERENCES

1) Yamada, O.: Coil planet centrifugation, an observation on seepage of cytosol substances from erythrocytes under continuously decreasing osmotic pressure. Kawasaki Medical Journal 2: 19-34, 1976
2) Shibata, S. and O. Yamada: Clinical laboratory tests by means of coil planet centrifugation. J. Med. Technol 20: 373-382, 1976
3) Millonig, G.: Further observations on a phosphate buffer for osmium solutions in fixation, Electron Microscopy, Fifth International Congress for Electron Microscopy. Academic Press (New York and London) vol. 2, p 8, 1962
4) Singer, S. J. and G. L. Nicolcon: The Fluid Mosaic Model of the Structure of Cell Membranes, cell membranes are viewed as two-dimensional solutions of oriented globular proteins and lipids, Science 175: 720-731, 1972
5) Fuji, T.: Notes on erythrocyte membrane. Protein Nucleic Acid Enzyme (Tokyo) 19: 52-72, 1972
6) Yawata, Y:. Red cell membrane and hemolysis -Effect of cyclic necleotides on membrane protein phosphorylation in human red cells. Metabolism and Disease 12 (4), 1975
7) Kawagoe, Y. and T. Enami: Separation of young and aged erythrocytes by coil planet centrifugation. Jpn. J. Clin. Pathol. 22 (supplement): 123-123, 1974
8) Kitazima, K. and S. Shibata: Coil planet centrifugation and its application to the observation of altered membrane properties of erythrocytes in hepatobiliary disorders. J. Lab. Clin. Med. 85: 855-864, 1975
9) Bull, B.: A simple Flow Chamber for Hydaulic Manipulation of Individual Cells, Red Cell Shape. Physiology. Pathology. Ultrastructure. Springer-Verlaf (New York, Heidelberg, Berlin) p 115-124, 1973