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COIL PLANET CENTRIFUGATION, AN OBSERVATION ON SEEPAGE OF CYTOSOL SUBSTANCES FROM ERYTHROCYTES UNDER CONTINUOUSLY DECREASING OSMOTIC PRESSURE*

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Abstract

Coil planet centrifugation proviedes us with a new measure particularly suitable for observation of sequential alteration of the membrane of erythrocytes when they are exposed to a stress produced by a rectilinearly decreasing osmotic pressure of salt solution at a constant speed. The osmotic gradient is prepared in a long slender polyethylene coiled tube ("coil") which serves as a centrifuge tube.

Venous blood was taken from 65 normal adult subjects, Anticlot ET was added to prevent coagulation, and a 10 μl aliquot of blood was applied to a "coil", sealed and centrifuged. The erythrocytes migrated from the higher to the lower osmolar side of the coil, and hemolyzed at the sites where they could no longer comply with the osmotic stress. The coil (3 m) was cut into 15 pieces of equal length and their contents were analyzed for the chemical components leaked out of erythrocytes through the injured membrane: potassium ion (MW 39), reduced glutathione (MW 307), hemoglobin (MW 64,500), glutathione reductase (MW 115,000). Erythrocyte ghosts were also counted.

The results of this experiment disclosed a sequence of release of components from erythrocytes, K^+ (the fastest), GSH, hemoglobin, and GSSG-R (the slowest), in the order listed, and this was followed by the appearance of ghosts.

This release sequence suggested that the smaller is the intraerythrocytic substance in molecular size the faster leaks the relevant substance from the erythrocytes.

INTRODUCTION

Until recent days dynamic aspects of the process of hemolysis of erythrocytes exposed to hypotonic milieu have been left little explored.

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A few investigators, however, challenged the obscurity of this process. Marsden and Östling (1959)[1] were interested in the mechanism of outward release of the materials contained in erythrocytes through their membrane into the ambient medium which was made variously hypotonic with dextran. They noted that dextran of lower molecular weight invaded the erythrocytes more easily than that of higher molecular weight. Hjelm, Östling and Persson (1966)[2] analyzed partially hemolyzed solutions produced by placing erythrocytes in variously hypotonic medium for several enzymes as well as hemoglobin, and pointed out that enzymes of smaller molecular size were discharged from the erythrocyte earlier than those of larger molecular size. MacGregor II (1972)[3] also mentioned that the difference in the attitude of movement of the substances contained in rat erythrocytes during hemolytic process depended on molecular sizes.

Coil planet centrifuge which had been invented by Nunogaki and Ito (1966)[4] was recently remodelled and improved in efficiency[5,6,7,8]. It has become an instrument particularly suitable for the observation of dynamic hemolytic process of human erythrocytes which are exposed to an incessantly changing osmotic stress by making them migrate by centrifugal force in a coiled path ("coil") of salt solution of rectilinear osmotic gradient, starting from the site of higher osmolarity (120 mOsM) to that of lower osmolarity, with a constant speed (0.5 cm/sec). The hemolysis which takes place in the "coil" is not static like the one seen in the test tubes of salt solutions of discretely graded osmolarity which are arranged in Parpart's procedure[9]. In this procedure erythrocytes are exposed to a constant hypotonicity which does not change from the beginning to the end of hemolysis, only being dependent on the concentration of the salt solution put into the relevant individual test tubes. The experimental conditions set up by Marsden and Östling[1], and Hjelm, Östling and Persson[2] were also static.

How does human erythrocyte undergo changes, and how are the substances contained and stored in human erythrocyte released through its membrane to the outside when it is subjected to dynamic osmotic stress by coil planet centrifugation? In our experiment a design was made to answer this question. Substances of different molecular sizes belonging to human erythrocytes, including potassium ion, reduced glutathione, hemoglobin and glutathione reductase, and erythrocyte ghosts in the hemolyzed solutions obtained from "coil" were analyzed or counted.

The purpose of this paper is to present the results of our observation of dynamic osmotic hemolysis of human erythrocytes by coil planet centrifugation, in comparison with those of the study on the static hemolysis by Parpart's procedure.

MATERIALS AND METHODS

1. Coil planet centrifugation[8] (Fig. 1): In coil planet centrifugation

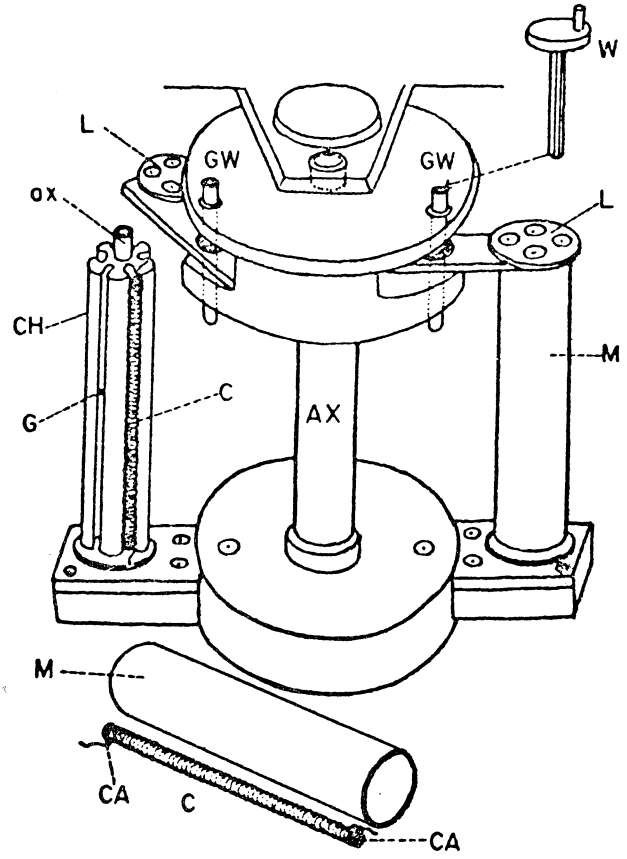

Fig. 1. Outline of the structure of CPC
AX: main axis for revolution. ax: the second axis for rotation. C: coil. CA: cap put at the end of a coil. CH: coil holder removed of its mantle. G: groove of a coil holder into which coils are inserted. GM: guide canal for the wrench which is employed to fix a lid at the top of a coil holder. L: lid of a coil holder. This serves as a wing fixing a coil holder to main axis. M: mantle case for covering a coil holder as a whole. W: wrench used for fixing a coil holder to the main axis. (Kitazima and Shibata 1975)

one employs a "coil" as a centrifuge tube. The coil is a long slender polyethylene tube (bore diameter, 0.3 mm and length, 3 m) which is tightly wound round a plastic stick (diameter, 5 mm and legth, 20 cm) in its middle 17 cm portion leaving its both ends free. This is equivalent to an unusally long sedimentation tube having a depth of 3 m, if ordinary centrifugation is thought of.

In this coil a column of saline solution with a rectilinearly decreasing osmotic gradient from 120 mOsM to 50 mOsM is packed by means of a gradientor (Sanki Engineering Ltd., GRD-V). The saline (pure NaCl) solutions (120 mOsM and 50 mOsM) are checked beforehand for their osmolarity by an osmometer.

An aliquot of 10 μl of blood sample which has been prevented from coagulation with Anticlot ET (1 drop to 1 ml blood) is applied to the 120 mOsM end of the coil. The coil is sealed by heat with soldering iron at both ends (120 mOsM and 50 mOsM), and incubated at 37°C in a warm box for ten minutes. It is put into the coil planet centrifuge (CPC) and centrifuged (revolution 1600 RPM, self rotation 16 RPM) for ten minutes (at 37°C)

The erythrocytes of the blood sample, namely the particles suspended in the fluid column of the coil, are driven from the higher osmolar portion (120 mOsM) to the lower (50 mOsM) with a constant speed (0.5 cm/sec) while the coil is making revolution round the principal axis of CPC simultaneously with self rotation round its own axis in the same way as the earth (a planet) makes revolution around the sun. The erythrocytes are exposed to a continuously increasing hypotonic stress while they are driven forth until they come to the sites where they can no longer comply with the stress and hemolyzed, giving rise to a hemolysis band in a certain part of the coil.

At the end of centrifugation the coil is removed from the CPC, untied from its stick little by little (in accordance with 10 pitches of the coil), and its whole length is cut into 15 equal pieces. The solution contained in each part is transferred into a cup separately.

The solution contained in each piece is counted for the number of erythrocyte ghosts (per mm^3). After it has been centrifuged (2500 RPM for 15 minutes), its supernatant is analyzed for potassium ion (K^+; molecular whight, 39), reduced glutathione (GSH; MW, 307), hemoglobin (Hb; MW, 64,500) and glutathione reductase (GSSG-R; MW, 115,000).

2. Materials: Venous blood was withdrawn from the antecubital vein of 65 normal adult subjects, and Anticlot ET was added (1 drop to 1 ml

of blood) to prevent coagulation. The blood sample was employed for examination within a day after its collection.

3. Determination of potassium concentration: An aliquot of 20 μl of the solution taken from the coil displaying a "hemolysis band" was added to and mixed with 4.0 ml of diluent solution containing lithium salt in a certain specified concentration in order to be nebulized and atomized in a flame photometer (Radiometer Co.) for reading K^+ concentration (mEq/l). Simultaneously, 5 μl of blood plasma separated from the blood of the same subjects was applied to a coil, sealed and centrifuged in the same way as the blood sample. The coil was cut into pieces of equal length, and their contents were measured for potassium ion, so that the contamination with coexistent plasm K^+ may be eliminated by substraction of K^+ values of the "plasma" coil from the K^+ estimation of the "hemolysis band" coil.

4. Estimation of GSH concentration[10]: Metaphosphoric acid solution (600 μl) was added to the "hemolysis band" solution (400 μl), mixed, allowed to stand for ten minutes, and centrifuged (3000 RPM for 5 minutes) to separate the top layer. This was repeated once more and the top layers were collected. To the collected top layer solution (500 μl) 1.0 M phosphoric acid buffer solution of pH 8.0 (500 μl) and color reagent DTNB [0.001 M, 5,5′ Dithiobis-(2-nitrobenzoic acid) solution, 250 μl] were added and mixed. Exactly one minute later the absorbance of the solution (optical path 1.0 cm) OD was read at 412 nm. The concentration of GSH was expressed in $OD_{412\ nm}$.

5. Determination of hemoglobin (Hb) concentration: Hemokit N (Nippon Shoji) coloring reagent (A neutral solution of potassium ferricyanide and potassium cyanide cotaining a detergent) was diluted fifty times with distilled water. To 2.5 ml of this solution was added 10 μl of solution taken from the coil ("hemolysis band") to convert Hb into CN-met-Hb. The absorbance of the mixture at 420 nm (Soret band) was measured and Hb concentration was read by collating the absorbance against the calibration curve which was constructed with hemoglobin solutions of known concentrations beforehand.

6. Measurement of GSSG-R activity: Long and Carson's procedure[12] was followed. Glutathione (GSSG), NADPH and the sample (content of the "hemolysis band" coil) were incubated at 37°C in a cuvet in a Gilford 2400 spectrophotmeter to pursue the decreasing absorbance of the mixture at 340 nm, which represented the reduction of GSSG to GSH by GSSG-R at the expense of NADPH possessing light absorption

in the ultraviolet region (340 nm). GSSG-R in the sample had been activated by addition of FAD to convert all the inactive enzyme into the activated form beforehand[13] (10 μM of FAD was added to 100 μl of solution taken from the coil with "hemolysis band", and warmed at 37°C for 30 minutes). The enzyme (GSSG-R) activity was expressed in terms of the magnitude of descent of the absorbance ($\Delta OD_{340\ nm}$) for 18 minutes.

7. Count of ghosts: The solution contained in the coil (with "hemolysis band") was transferred into a cup and it was diluted twenty times with physiological saline solution in a mélangeure (for white cell counting). The diluent was taken in a counting chamber, and counted for the ghosts under a phase contrast microscope in the same way as the conventional white cell counting. The count was expressed in terms of the number of ghosts per cubic millimeter.

8. Osmotoc fragility test by Parpart[9]: Mother solution of 10 % NaCl-phosphate buffer (pH 7.4) was diluted with distilled water to get 1 % salt solution, and this was diluted again with water so that a series of hypotonic saline solutions (0.55, 0.50, 0.488, 0.475, 0.463, 0.45, 0.438, 0.425, 0.413, 0.40, 0.375, 0.35, 0.30, 0.25, 0.10 % NaCl) might be obtained. Aliquots of 2.0 ml of these hypotonic saline solutions were introduced into centrifuge tubes individually. Volumes of 100 μl of venous blood prevented from coagulation were added to them, and mixed. The mixtures were left at room temperature for 30 minutes and centrifuged (2000 RPM, for 5 minutes) to separate the top layers which represented hemolysis. The absorbance of the solutions of the top layers were measured in a spectrophotometer at the wave length of 500 nm (optical path 1.0 cm) against a blank consisting of 0.1 % NaCl solution. The degree of hemolysis was expressed by "per-cent hemolysis", namely the absorbance of the top layer salt solution of the relevant tube ÷ absorbance of the top layer solution of 0.1% NaCl tube $\times 100$ %.

The top layer solutions were also measured for potassium concentration in a flame photometer in the same way as described in 3. The values of potassium estimations were referred to as "potassium release", which represented the potassium ions leaking out of erythrocytes in the process of hemolysis. The "per-cent hemolysis" (new hemolysis) and the "potassium release" (new K^+ liberation) which actually occurred due to the hypotonicity pertaining to a certain specified tube were calculated by subtraction of the "per-cent hemolysis" and the "potassium release" of the preceding tube from those of the relevant tube.

RESULT

The resuts obtained in this investigation are presented in Figs. 2, 3, 4, and 5. These Figs. depict graphically the average values of analyses of K^+, GSH, GSSG-R and hemoglobin in the 15 sequentially excised segments of the coils in which blood samples from 20 normal subjects were subjected to hypotonic stress (120 mOsM → 50 mOsM). The ordinates refer to concentrations or activities of the intraerythrocytic substances or enzymes, while the abscissae to the osmolarity of the solution contained in the segments. Broken lines represents the ratio of the analysis values of individual substances to the concentration of hemoglobin which was regarded as standard substance representing the degree of hemolysis.

As shown in Fig. 2, hemoglobin (molecular weight 64,500) is released from the erythrocytes in a way that makes a bell-shaped histogram: hemolysis starts at the 6th segment from the higher osmolarity terminus

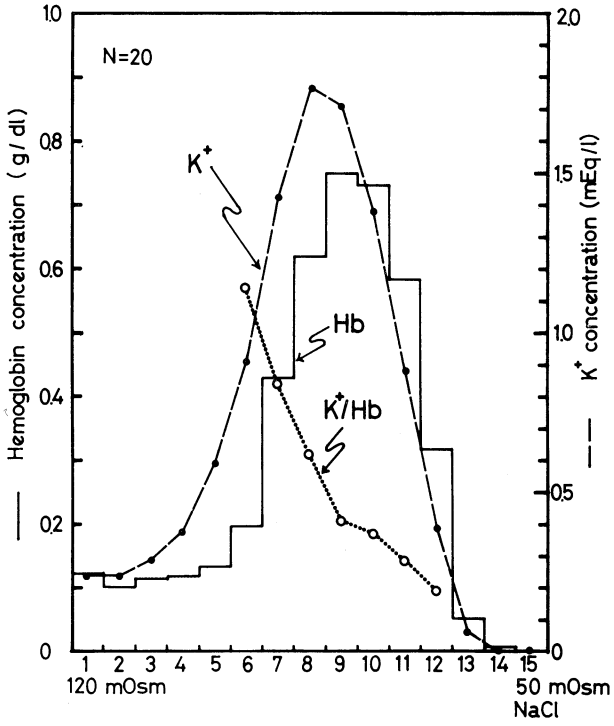

Fig. 2. Seepage of potassium (K^+) from erythrocytes (broken line) as compared with leakage of hemoglobin from them (Hb: histogram) in the process of dynamic osmotic hemolysis by coil planet centrifugation.

(120 mOsM) of the coil, and ends at the 13th segment, with a peak at the 9th segment. The histogram is not perfectly symmetrical in shape, with a rather gentle, ascending limb and a steep descending limb. The release curve (broken line in the Fig.) of potassium ion K^+ (molecular weight 39) resembles that of hemoglobin, but release starts earlier (at the 3rd segment) making a peak (the 8th segment) at a position slightly shifted to the higher-osmolar side than that of hemoglobin. Accordingly the curve is shifted to the higher-osmolar side as a whole.

Scrutiny of the curve and the histogram (K^+ and hemoglobin) in comparison with the curve of K^+/hemoglobin ratio which is drawn in a dotted line will lead us to recognize that in the ascending limb potassium ion is released earlier and faster than hemoglobin, and its release declines progressively in speed and amount in the descending limb, at length terminating in the 14th segment in which hemoblobin is still in the process of leakage from erythrocytes.

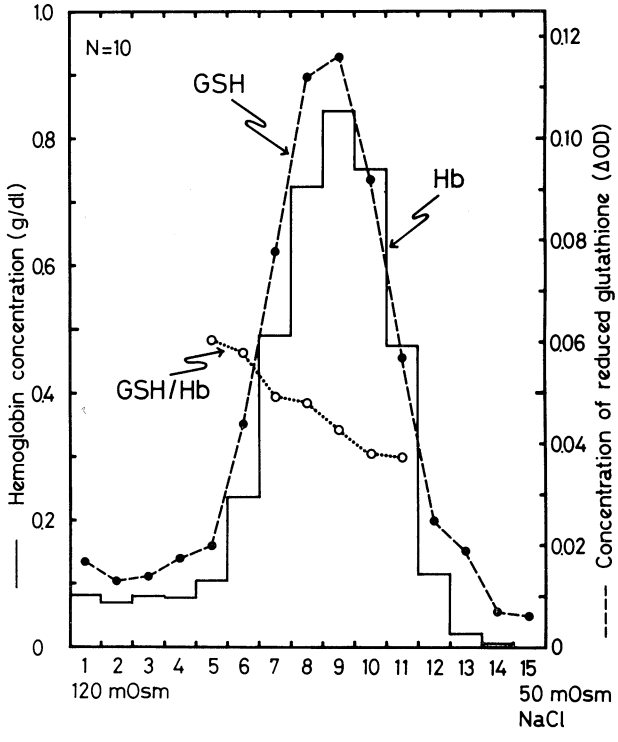

Fig. 3. Leakage of reduced glutathione (GSH; broken line) and hemoglobin (Hb; histogram) from erythrocytes in the process of dynamic osmotic hemolysis by coil planet centrifugation.

Fig. 3 illustrates the process of the seepage of GSH (molecular weight 307) (broken line). In this case the overall lineation of the GSH seepage curve is slightly shifted to the higher osmolar side of the hemoglobin release curve. The GSH/Hb ratio (dotted line) makes a straight line running from top-left to bottom-right gently. The inclination is significant, but it is not so salient as seen in the case of K^+/Hb ratio line. This indicates that GSH leaves erythrocyte through its membrane to the external milieu after potassium ion, but it goes ahead of hemoblobin leakage.

GSSG-R (molecular weight 115,000) is twice as large as hemoglobin (molecular weight 64,500) in molecular size. Fig. 4 shows its release curve from erythrocytes (broken line). This curve is coincident almost completely with hemoglobin release curve. The ratio of GSSG-R to hemoglobin gives a holizontal straight line in the region except for a small part lying in the higher osmolar side. It is therefore thought

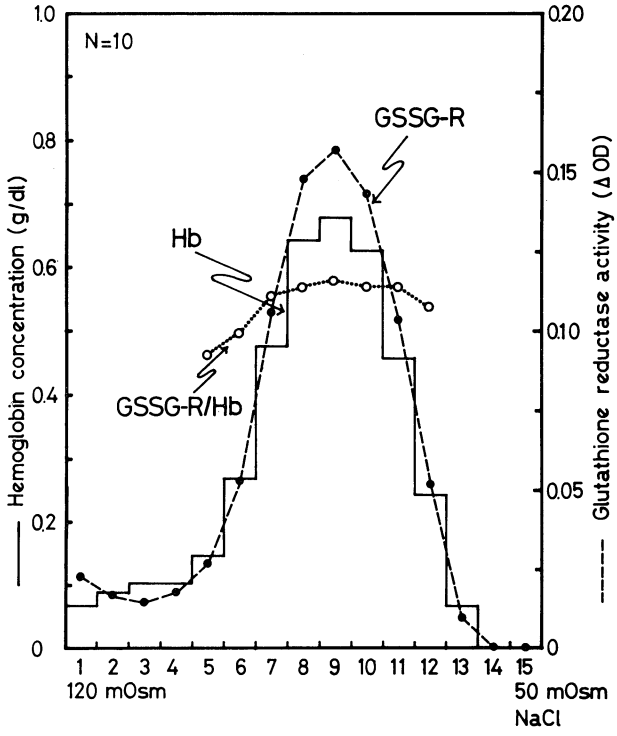

Fig. 4. Leakage of glutathione reductase (GSSG-R; broken line) hemoglobin (histogram) in the process of dynamic osmotic hemolysis by coil planet centrifugation.

that there is no significant difference between hemoglobin and GSSG-R with respect to leakage from erythrocyte.

The distribution of the ghosts in the coil is drawn in comparison with hemoglobin release curve in Fig. 5. The peak of ghost distribution curve (a broken line) is seen at the 9th segment, just on the same point as the summit of hemoglobin release curve. However, the over-all aspect of the ghost distribution curve is suggestive of its slight shift to the lower osmolar side over the hemoglobin release curve (histogram). Its ascending limb is rather gentle and the descending limb is fairly steep. This may connote that ghosts are slightly dislocated from the place where relevant erythrocyte has been hemolyzed completely by centrifugal force.

It is worthy of mentioning that the ratio of ghost to hemoglobin ascends from the bottom-left (higher osmolar side) toward the top-right side (lower osmolar side). This indicates that the ghosts of erythrocytes after completion of hemolysis act as gigantic particles to which centri-

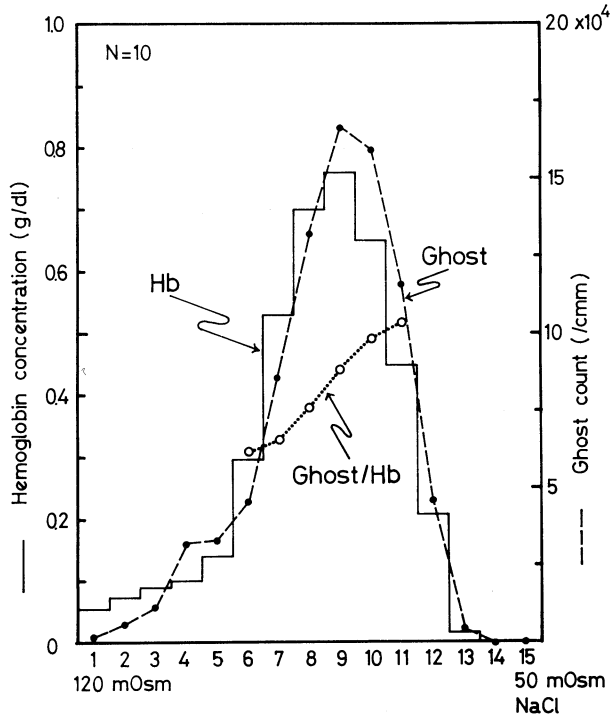

Fig. 5. Leakage of hemoglobin (Hb; histogram) and ghost count (broken line) in the process of dynamic osmotic hemolysis by coil planetcentrifugation.

fugal force exerted by CPC is sufficiently effective. Hence, they are dragged and dislocated from the hemolysis site to the distal direction, namely to the side of lower osmolarity in the coil.

Fig. 6 shows the result of a similar experiment of hypo-osmolar hemolysis carried out by Parpart's procedure. Solid line refers to hemoglobin release, and broken line to potassium ion release. It will be seen in this figure the peak of potassium ion release situates in the position slightly left (higher osmolar side) to that of hemoglobin peak. The disagreement between the two curves is more salient in the right (lower osmolar) side than in the left (higher osmolar) side. This makes a contrast to the result obtained by CPC. In Parpart's procedure the ghost distribution curve (dotted line) and the per-cent hemolysis curve (solid line) are actually identical as seen in Fig. 7.

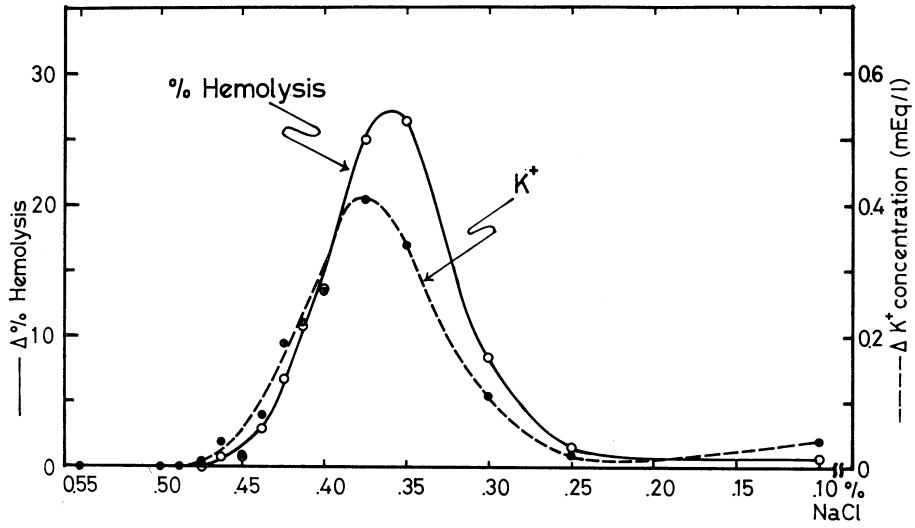

Fig. 6. Leakage of potassium (K^+) from erythrocytes in comparison with hemolysis in Parpart's osmotic fragility test of erythrocytes.

DISCUSSION

Parpart's test for Osmotic fragility of erythrocytes[9] examines the hemolytic process which occurs in a erythrocyte suspension prepared by addition of a certain volume of blood samples to a certain amount of hypotonic salt solutions that were serially diluted. The observation in this test concerns essentially with inspection of erythrocytes which are deformed or changed under a constant osmotic stress of a certain degree for a certain period of time. The environment in which ery-

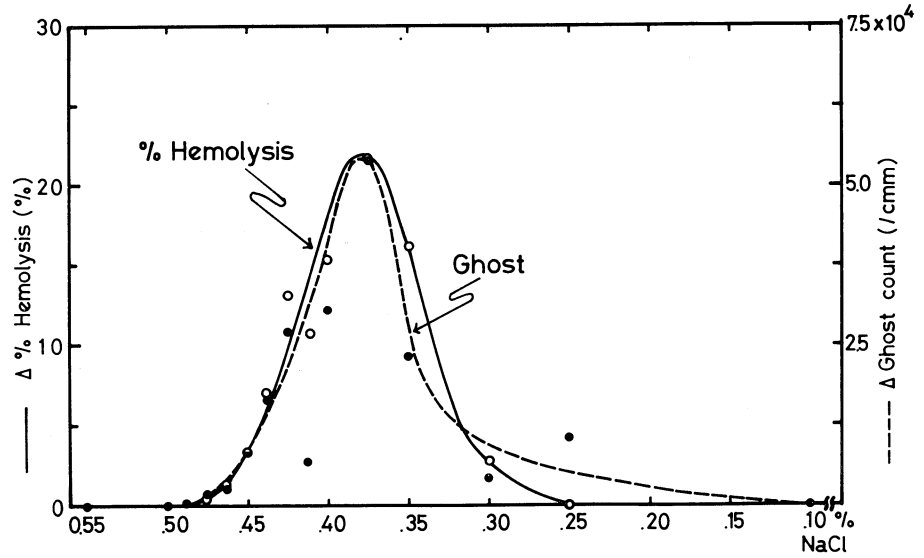

Fig. 7. % hemolysis in comparison with ghost count in Parpart's osmotic fragility test.

throcytes are placed is therefore static in the individual test tubes of suspensions. In Parpart's procedure the amount or concentration of hemoglobin in an optional test tube in the series represents the cumulative hemolysis occurring in and prior to that tube. Therefore, the new hemolysis pertaining to hypotonicity of that tube is calculated by subtraction of the concentration of hemoglobin in the preceding tube from that of the optional tube. This is referred to as "per-cent hemolysis" in this paper. In quite a similar way "potassium ion release" is computed. Fig. 6 illustrates the comparison of the per-cent hemolysis curve with the potassium ion release curve in relation to osmolarity. It is seen in this figure that both of these curves are bell-shaped and symmetrical, and the potassium ion release curve distinctly precedes the per-cent hemolysis curve in the descending limb region, whereas discrepancy between them is not significant in the ascending limb region. This will be interpreted as follows: In the static hypo-osmolar environment of Parpart's procedure in the test tubes containing salt solutions of comparatively high osmolarity (0.50-0.40 % NaCl), only the erythrocytes whose membrane is wasted by age are hemolyzed releaseing hemoglobin as easily as potassium. In contrast, in the test tubes or relatively low osmolarity younger erythorcytes with wholesome membrane undergo hemolysis. They resist against releasing hemoglobin more stoutly than

against liberation of potassium ion. The distribution curve of the ghosts (Fig. 7) is also symmetrical, just like the per-cent hemolysis curve.

On the contrary, in the dynamic osmotic fragility test as carried out by CPC, erythrocytes are rolled over from the higher osmolar side to the lower osmolar side and hemolyzed by cumulative effect of continuously increasing hypotonic stress.

In this case (Fig. 2), the K^+ release and the Hb release curves show a gently ascending limb and a steeply descending limb. The erythrocytes leak potassium ion which is smaller in molecular size more easily than hemoglobin which has larger molecule while being rolled down from the higher osmolar side (120 mOsM) toward the lower osmolar side (50 mOsM), forming the peaks of their release curves in the relatively higher osmolar region. Consequently, K^+ release curve precedes Hb release curve more saliently in the higher osmolar region than in the lower osmolar. Thus the two curves are more diffrent and apart from each other in the ascending limbs. It is worthy of mention that, in Fig. 2, the ratio of K^+ to Hb makes a rectilinear line steeply descending from top-left toward bottom-right.

This indicates that potassium ion is discharged from erythrocytes more rapidly and more abundantly than hemoglobin in the early part of hemolysis (ascending limbs) and its stock in erythrocytes is consumed faster than that of hemoglobin, so that in the later part of hemolysis (descending limbs) the potassium ion released from erythrocytes becomes less and less in comparison with hemoglobin.

Fig. 3 shows the similar aspect seen in GSH/Hb ratio. However, the slope of the ratio line, which runs rectilinearly from top-left down to bottom-right, is not so steep as K^+/Hb ratio line. It is interesting that the ratio of GSSG-R to Hb rises from the bottom-left to top-right in the early part and reaches plateau, forming a horizontal line. This is suggestive of that, on the higher osmolar side, GSSG-R leaks from erythrocytes less at first, but slightly later, when erythrocytes migrated toward the lower osmolar side to some extent, GSSG-R and hemoglobin leave erythrocytes at the same pace and speed.

The ghost to Hb ratio line ascends rather steeply from bottom-left to the top-right as shown in Fig. 5. This indicates that erythrocyte ghosts continue migration toward the lower osmolar direction to a certain extent from the sites where erythrocytes are hemolyzed completely under the influence of centrifugal force, although hemoglobin which is too small in molecular size to be affected by centrifugal force

remains at the place where it was released from erythrocytes. If this is really the case, it will be germane to presume that when erythrocytes are exposed to hypo-osmolar stress the intraerythrocytic materials escape from the erythrocytes in a sequential order in reverse of molecular size, i.e., $K^+ \rightarrow GSH \rightarrow Hb \rightarrow GSSG\text{-}R$. MacGregor II[3] who mixed rat erythrocytes with isotonic and hypotonic solutions in a mixing chamber, thus decreasing osmolarity gradually to observe hemolytic process, obtained the results similar to ours. Recently Hjelm, Östling and Persson[2] suspended washed human erythrocytes in Hendry's saline dextran solution[14] series to hemolyze them. They analyzed intraerythrocytic ingredients liberated from erythrocytes to the top layer of solutions following centrifugation. According to their investigation the fastest in escaping from erythrocytes were ATP and GSH. They were released through the erythrocyte membrane ahead of hemoglobin. Hemoglobin was in conjunction with G-6-PDH (glucose-6-phosphate dehydrogenase) and hexokinase. Catalase and LDH (lactate dehydrogenase) were liberated from erythrocytes after hemoglobin. The sequential order of releasing was inversely related to the molecular size of the individual intraerythrocytic components, and there was no indication of their adherence to erythrocyte membrane that may hamper their free leakage.

Since, in the CPC procedure, erythrocytes of blood sample are exposed to increasing hypotonic stress in natural state without washing, it is thought that the results obtained in our investigation mirrors the attitude of erythrocytes against hypotonicity more strainghtforwandly than in the procedure employed by Hjelm and his associates[2].

What kind of structural alteration of cell membrane is related to the release of intraerythrocytic components which are placed against a continuously enforced hypotonic stress? Are submicroscopic pores are produced through the erythrocyte membrane by hypotonic stress? Are they increased in number with increasingly strengthened hypo-osmolarity? Do the intraerythrocytic components seep through the pores to the outside of erythrocytes in increasing amount with the growing number of pores? Are crevices created in the cell membrane at extremely low level of osmolarity? Do the intraerythrocytic components escape freely and in abundance through the crevices? In fact, Seeman and his associates[15], who studied electron-micrcscopically the erythrocytes exposed to hypo-osmolarity, stated that fissures of about 1 mμ length were seen all over the cell membrane and they grew larger with the decrease of the osmolarity of ambient medium. Bessis[16], who em-

ployed a scanning electron microscope for the observation of the same subject, obtained another result. According to him discoid erythrocytes are transformed into echinocytes with thorny cell membrane in hopo-osmolar medium and through the thorns are released the intraerythrocytic components.

According to recent investigations[17] erythrocyte membrane is thought to be composed of double laminar layers (outer and inner) of lipid molecules (cholesterol and phospholipid) which are not rigid but viscid. These lipid double layers are scatteringly penetrated perpendicularly by protein columns which connect the inside with the outside of the cell partially or completely. It is said that when erythrocytes are placed in a hypotonic saline solution water flows into the cell through the protein columns which permits the passage of water, and the ingredients contained in the cell leak though the protein-columns which are different from those serving as pathy-way for water. Thus erythrocytes swell until their surface areas become 110 per cent as large as in original state, and at this extreme point they are hemolyzed.

In this paper our discussion is confined to mentioning the fact that in dynamic osmotic fragility test of erythrocytes as observed by CPC the intraerythrocytic component seep through their membrane to the outside with the speed inversely proportional to their molecular sizes, namely the smaller the molecular size of an intraerythrocytic component the faster does the relevant component spill out of the erythrocyte.

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REFERENCES

1) Marsden, N. V. D. and Östling, S. G.: Accumulation of dextran in human red cells after haemolysis. Nature 184: 723-724, 1959
2) Hjelm, M., Östling, S. G. and Persson, A. E. G.: The loss of certain cellular components from human erythrocytes during hypotonic hemolysis in the presence of dextran. Act Physiol Scand 67: 43-49. 1966
3) MacGregor, II R. D. and Tobias, C. A.: Molecular sieving of red cell membranes during gradual osmotic hemolysis. J Membrane Biol 10: 345-356, 1972
4) Ito, Y., Weinstein, M. A., Aoki, I., Harada, R., Kimura, E. and Nunogaki, K.: The coil planet centrifuge. Nature 212: 985-987, 1966
5) Ito, Y., Aoki, I., Kimura, E., Nunogaki, K. and Nunogaki, Y.: New microliquid-liquid partition techniques with the coil planet centrifuge Anal Chem 41: 1579-1584, 1968

6) Shibata, S., Kobayashi, K. and Sera, Y.: Application of coil planet centrifugation to hematology,—measurement of osmotic fragility of erythrocytes and autohemolysis test. J Japan Med Instum 40: 619-626, 1970
7) Shibata, S. and Miwa, S.: Coil planet centrifugation as applied to the diagnosis of hemolytic anemias. Abstructs, XIV International Congress of Hematology (São Paulo July 16-21) 1972 (# 17)
8) Kitazima, K. and Shibata, S.: 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) Dacie, J. V. and Lewis, S. M.: Practical Haematology, 4th Ed. Churchill, London, 1968
10) Beutler, E., Duron, O. and Kelly, B. M.: Improved method for the determination of blood glutathione. J Lab & Clin Med 61: 882-888, 1963
11) Matubara, T., Okuzono, H. and Tamagawa, S.: Proposal for a improved reagent in the haemiglobincyanide method, Modern Concept of Hematology, Academic Press, New York/London, 1971, P29-43
12) Yunis, J. J.: Biochemical Methods in Red Cell Genetics, Academic Press, New York/London, 1969, p155-163
13) Staal, G. E., Visser, J. and Veeger, C.: Purification and properties of glutathione reductase of human erythrocytes. Biochim Biophys Acta 185: 39-48, 1966
14) Hendry, E. B.: Osmolarity of human serum and of chemical solutions of biological importance. Clin Chem 7: 154-164, 1961
15) Seeman, P., Cheng, D. and Iles, G. H.: Structure of membrane holes in osmotic and saponin hemolysis. J Cell Biology 56: 519-527, 1973
16) Bessis, M.: Corpuscles, Atlas of Red Blood Cell Shapes, Springer Berlin/Heidelberg/New York, 1974
17) Singer, S. J., and Nicholson, G. L.: 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