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DISK TO SPHERE TRANSFORMATION OF ERYTHROCYTES, INDUCED BY 1-ANILINO-8-NAPTHTHALENESULFONATE

SHIGEO YOSHIDA* and AKIRA IKEGAMI

Department of Physics, Faculty of Science, University of Tokyo, Tokyo (Japan)

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SUMMARY

1. The agent 1-anilino-8-naphthalenesulfonate (ANS) has been found to induce a transformation of bovine erythrocyte from a biconcave disk to a smooth sphere through a crenated sphere.

2. The process of the transformation was observed with a scanning electron microscope. With the increase of ANS concentration crenations became steeper and more numerous at first, and the erythrocyte finally became spherical with a smooth surface.

3. The transformed erythrocyte was returned reversibly to the original shape by removing ANS as far as observed under an optical microscope.

4. Hematocrit measurements showed that the volume of the erythrocyte did not change throughout the transformation. This indicates that the surface area decreased by 10 % at the final stage.

5. Coulter counter measurements suggest that crenations are not removed but flatten out in the process from crenated sphere to smooth sphere.

INTRODUCTION

Normal mammalian erythrocytes are known to have the shape of a biconcave disk. Their shape becomes a crenated disk, a crenated sphere, and a smooth sphere in succession, either by the addition of amphiphilic–anionic agents, by an increase in pH, or by lowering the ATP level in erythrocytes (crenated type transformation). On the other hand their shape becomes a shallow cup, a deep cup, and a smooth sphere in succession, either by the addition of amphiphilic–cationic agents or by a decrease in pH (cup type transformation) [1–3]. Since erythrocytes have not any subcellular structures, these morphological changes should be attributed to a structural change of the cellular membrane of erythrocyte.

It was found by the authors that a crenated type transformation is induced by

Abbreviation: ANS, 1-anilino-8-napthalenesulfonate.

* Present address: National Institute of Industrial Health, 2051, Kizukisumiyoshi-cho, Kawasaki, Kanagawa, Japan.

an amphiphilic-anionic fluorescence probe 1-anilino-8-napthalenesulfonate (ANS). The use of this probe offers special advantage because its fluorescent properties are sensitive to environmental change [4]. Moreover, it was reported that the binding of this agent affects the permeability of the erythrocyte membrane for ions [5]. Thus, ANS can be used not only as a fluorescent probe for conformational change but also as an agent for the crenated type transformation or functional changes of the membrane by itself.

In the present work the crenated type transformation induced by ANS was studied extensively in an attempt to clarify the action of ANS on the membrane.

MATERIALS

The agent ANS was purchased from Eastman Organic Chemicals Co., and recrystallized twice [6].

Fresh bovine blood was anticoagulated with acid–citrate–dextrose and erythrocytes were washed twice with 150 mM NaCl containing 5 mM sodium phosphate buffer, pH 7.0 (a standard salt solution). The standard salt solution was used for washing and suspending erythrocytes throughout the study.

METHODS

Scanning electron microscopy

A fixative solution was prepared as an aqueous mixture of glutaraldehyde, NaCl, ANS and sodium phosphate buffer (pH 7.0). In the mixture the concentration of glutaraldehyde was 2 weight % and that of ANS was varied according to the ANS concentration in the erythrocyte suspension. The osmotic pressure was made 310 ideal mosM [7] with NaCl and sodium phosphate [8].

The agent ANS was added to 10 ml of 5 % (by vol.) erythrocyte suspension. After 10 min 10 ml of the fixative solution was dropped little by little into the suspension for more than 30 min with gentle stirring. Then the erythrocyte was washed three times with a standard salt solution and dehydrated with acetone. The entire process was carried out at 4 °C. A droplet of the suspension was placed on a cover glass and allowed to dry in air. The specimen was coated with carbon and then with gold. The photographs were taken with JSM-U3 (Japan Electro Optics Laboratory).

Hematocrit measurements

An aliquot of 100 mM ANS solution, made isotonic with NaCl, was added to 3 ml of the 30 % erythrocyte suspension. 5 min after the addition of ANS a small portion of the erythrocyte suspension was drawn up into a microhematocrit tube. The tube was centrifuged at $10\,000 \times g$ for 15 min after sealing one end of it. The closest pack of erythrocytes was attained under this condition of centrifugation. The height of the total and the packed erythrocyte column were measured with a reading microscope. It was acertained with an optical microscope that the once packed erythrocytes remained in original shape when they were ejected from the microhematocrit tube after the centrifugation. At the same time the rest of the suspension was centrifuged at $300 \times g$ for 5 min and the extent of hemolysis was estimated from the optical absorbance of hemoglobin in the supernate at 543 nm. The hematocrit value was corrected for lysed erythrocyte volume from this estimation.

Coulter counter measurements

Sphered erythrocytes were fixed with glutaraldehyde in the same way as in the case of scanning electron microscopic observations omitting the process of dehydration. They were suspended in the standard salt solution. The mean cell volume of them was measured with a Coulter counter [9] Model B, a plotter Model H, and with a probe of 100 μm aperture diameter.

In order to test the sensitivity of the Coulter counter, erythrocyte suspensions of human, cow and sheep were used after they were made spherical with ANS and fixed with glutaraldehyde. Total volume and number of erythrocytes in the suspension was measured with the microhematocrit and the Coulter counter, respectively. Mean cell volumes were calculated from these values and compared with those calculated from the figures on the plotter of the Coulter counter [10]. The results are shown in Fig. 1. The response of the Coulter counter is directly proportional to mean cell volume while the accuracy of the Coulter counter measurement is better than 1 % from the repeated measurements.

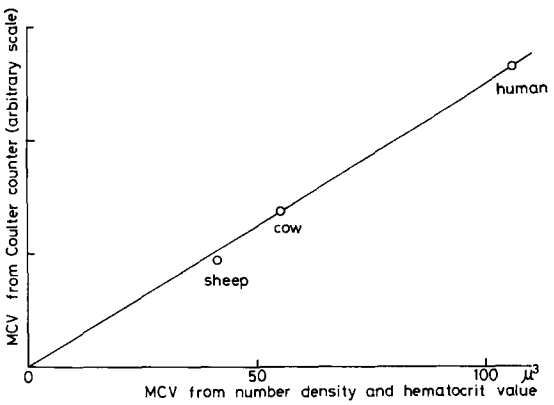

Fig. 1. Sensitivity test of the Coulter counter using the sphered erythrocytes of human, cow and sheep. The mean cell volume (MCV) calculated from the number density with the Coulter counter and the hematocrit value versus the mean cell volume from the figure on the plotter of the Coulter counter.

RESULTS

Optical microscopic observations

When ANS was added to the erythrocyte suspension, the shape of the erythrocyte changed to a crenated disk, a crenated sphere and a smooth sphere in succession with the increase of the ANS concentration up to 3 mM. The transformation had completed within 30 s and the transient stage of the transformation could not be observed. The transformed erythrocyte was stable and a further change of the shape was not observed for more than 30 min. The erythrocyte returned to the original shape by removal of ANS from the erythrocyte suspension. The second addition of ANS induced the same transformation as the first. This cycle could be repeated at least four times.

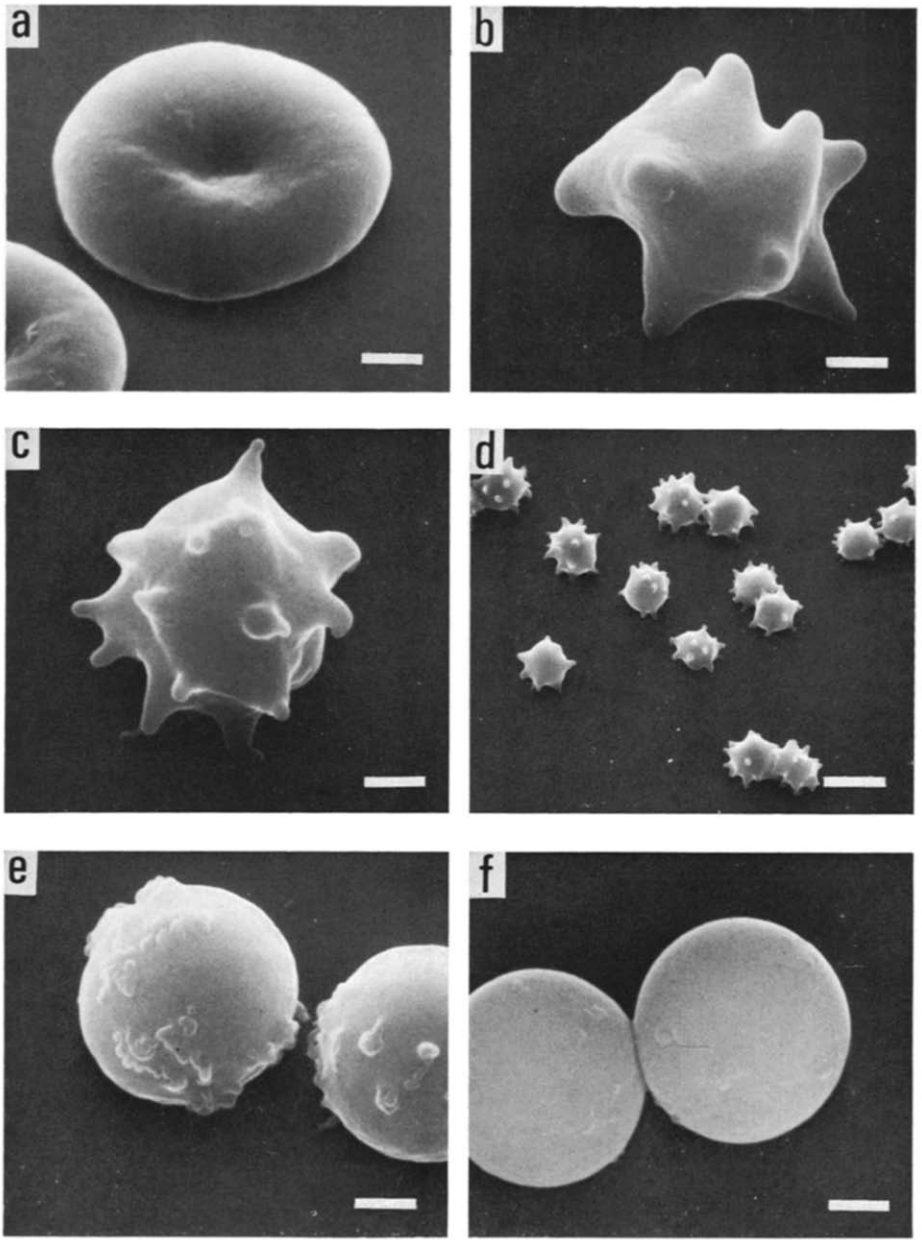

Fig. 2. Scanning electron micrographs of the erythrocytes transformed by ANS. a, ANS 0 mM; b, 0.03 mM; c and d, 0.1 mM; e, 0.7 mM; f, 3 mM. Bar represents 1 μm in a, b, c, e and f, and 5 μm in d.

Scanning electron microscopic observations

Fig. 2 shows some typical scanning electron micrographs of erythrocyte. Untreated erythrocytes had a biconcave disk shape with smooth featureless surface (Fig. 2a). At the ANS concentration of 0.03 mM, some of erythrocytes were transformed to a crenated shape (Fig. 2b) and others retained the biconcave disk shape. With the increase of the ANS concentration to 0.1 mM the biconcave disk disappeared and crenations became more numerous, longer and sharper (Figs 2c and 2d). As the concentration was increased further to 0.7 mM, crenations gradually flattened out (Fig. 2e). At 3 mM the erythrocyte became an almost smooth sphere with some traces of crenations on the surface (Fig. 2f).

There was considerable variation in number of crenations per erythrocyte at each ANS concentration. Fig. 3 shows the distributions of the numbers of crenations on an erythrocyte at each stage. Crenations which were not observed in the picture were estimated assuming the uniform distribution of crenations over the surface. Thus the total number of crenations on an erythrocyte, N_t, were calculated as follows: $N_t = 2N_0 - N_p$, where N_0 is the number of crenations which were observed in the picture and N_p is that of crenations whose tips protruded from the periphery. A few hundred of erythrocytes were examined at each concentration of 0.03, 0.05, 0.1 and 0.2 mM ANS. The crenations were longest at the concentration of 0.2 mM ANS. Data were omitted at the ANS concentration more than 0.2 mM because of the ambiguity in counting of crenations. The transformation proceeded accompanying the increase of the height and the number of crenations with the increase of ANS concentration. An erythrocyte whose crenations were less than six was seldom found.

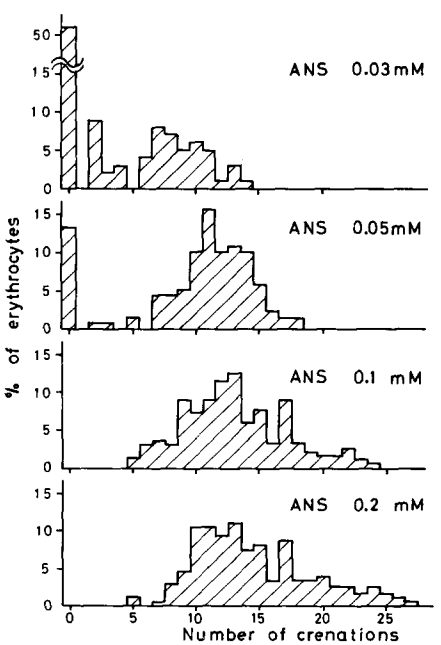

Fig. 3. Distribution of the number of crenations on an erythrocyte observed in photographs through the scanning electrons microscoscope.

The volume of the erythrocyte

The volume of the erythrocyte was measured by the microhematocrit method in each stage of the transformation. In Fig. 4 $100(H-H_0)/H_0$ is plotted against the ANS concentration, where H_0 and H are the hematocrit values at the zero and a certain concentration of ANS, respectively. A small amount of hemolysis was induced by the addition of ANS, and apparent volume decreased with the increase of ANS concentration as shown by the dashed line. Points in Fig. 3 are the experimental values corrected for hemolysis. There was no volume change throughout the transformation within 1 % of the experimental error.

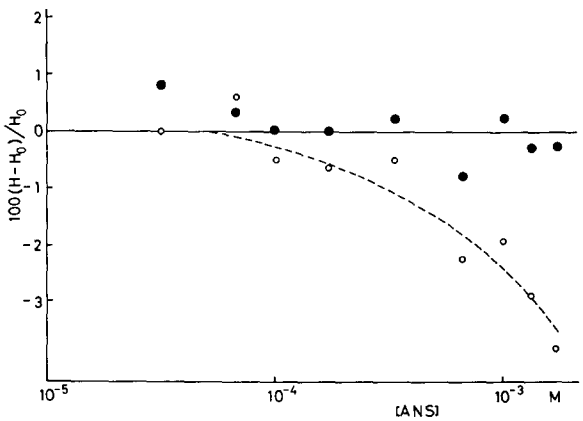

Fig. 4. Relative volume change of erythrocyte transformed by ANS. H_0 and H are hematocrit values at the zero and a certain concentration of ANS, respectively. ○, observed values; ●, corrected values for the volume of hemolysed erythrocytes.

Reversibility of the volume

The sharp crenations disappeared and the surface became smooth in the process of the transformation from crenated sphere to smooth sphere. In order to check whether the volume decreased irreversibly in the process, which might not have been observed by the microhematocrit method, the volume of erythrocyte was measured with the Coulter counter. At first erythrocytes were made smooth spheres by the addition of ANS to the final concentration of 3 mM. Then they were returned to biconcave disks by removal of the agent. Again they were made spheres as the first time. This cycle was repeated three times. Measurements were made of mean cell volumes of sphered erythrocytes both at the initial and final stage of these cycles after fixation with glutaraldehyde (Fig. 5). There was no difference between the mean cell volumes in the two states within the experimental error about 1 %.

DISCUSSION

In the process of the crenated type transformation, two different mechanisms are expected for the disappearance of crenations: The first is the separation and the second is the flattening out of crenations. The crenated form of erythrocyte may be approximated as a sphere of diameter 4 μm with 15 crenations of right circular cone

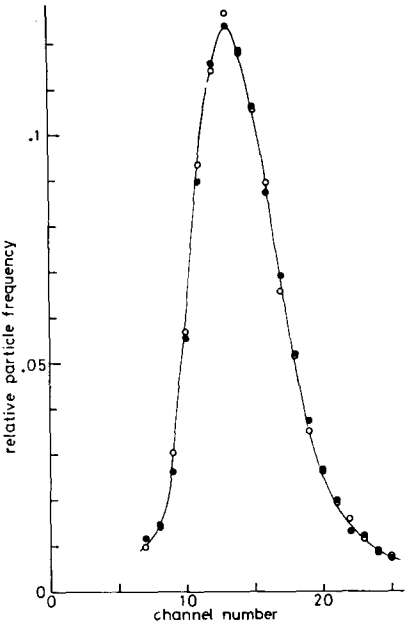

Fig. 5. Figures on the plotter of the Coulter counter of the sphered erythrocytes. ○, first sphered erythrocytes; ●, after the three cycles of the transformation.

(Fig. 3) whose diameter and height are 0.5 and 1 μm, respectively (Fig. 2c). If all the crenations separated from the erythrocyte, the total volume should decrease by about 3 % from the calculation with the above numerical values. There should be about 9 % decrease in volume after the three times repetition of the transformation, if all the crenations separated at each cycle from crenated sphere to smooth sphere. Whereas the Coulter counter measurements showed that the mean cell volume of sphered erythrocyte did not change more than 1 % after the three cycles of crenated transformation. Therefore it is concluded that crenations do not separate but flatten out. In the Coulter counter measurements sphered erythrocytes were fixed with glutaraldehyde in order to avoid the hemolysis due to the addition of ANS. But no deformation was observed after the fixation when examined under the optical microscope. Therefore, the fixation would not affect the mean cell volume measurement.

When erythrocytes are suspended in a hypotonic or a hypertonic solution, swelling or shrinking of the erythrocytes occurs with change in volume keeping the surface area constant. In the present transformation the surface area of an erythrocyte changes keeping the volume constant. An average size of biconcave bovine erythrocyte was found to be 6.3 μm in diameter, 1.0 μm in minimum width and 2.3 μm in maximum width on a magnified optical micrograph. The surface area and the volume were calculated to be 84 μm^2 and 61 μm^3, respectively, for the biconcave disk with above dimension [11]. When the erythrocytes were transformed from biconcave disk to sphere keeping the volume constant, the surface area should decrease by 10 %. It is impossible to calculate directly the surface area of the crenated erythrocyte, but it is most likely that the surface area increases in the intermediate state.

It appears that ANS acts on the membrane not with covalent bonds but with hydrophobic bonds in view of the fact that the transformed erythrocyte can be easily reversed to the original form by washing [4]. The process of transformation takes less than 30 s in the present case, whereas it takes more than half an hour in the case of saponin [1]. So the transformation shows wide variety in time depending on the agents.

The process of the transformation consists of two parts: the elongation and the flattening out of crenations. This suggests that there may be two types of binding sites of ANS molecules on the membrane. One of them corresponds to the elongation of crenations and the other to the flattening out. In fact there are two binding sites of ANS molecules to the ghost membrane: One of them may consist of protein (Site I) and the other of lipid or protein–lipid complex (Site II) (Yoshida, S. and Ikegami, A., unpublished). One reasonable model of the transformation is as follows. When ANS molecules bind to Site I the membrane expands and crenations grow in order to keep the volume of erythrocyte constant. When ANS molecules bind to Site II the membrane shrinks and crenations flatten out.

The crenated type transformation was observed for human, horse and sheep erythrocytes as well as bovine erythrocytes by ANS, regardless of the variety of lipid and protein composition in membranes [12]. So the transformation may be derived from the nonspecific character of the biological membrane.

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