A rapid micro method for recording red cell osmotic fragility by continuous decrease of salt concentration

D. DANON

From the Polymer Department, The Weizmann Institute of Science, Rehovoth, Israel

SYNOPSIS A single volume of 0.075 ml. of a 1 in 10 dilution of whole blood in isotonic NaCl solution is introduced into a container cell, two walls of which are made of a dialysing membrane. The container cell is introduced into a test tube of distilled water, placed in an instrument which is essentially a colorimeter with a recorder, between the source of light and the photoelectric cell. Dialysis through the membrane results in a continuous decrease in the salt concentration of the medium surrounding the erythrocytes. The measurement of the degree of haemolysis is based on the increasing transparency of the erythrocyte suspension while haemolysis takes place. Recording this increasing light transmission as a function of time, *i.e.*, as a function of decreasing salt concentration, yields the osmotic fragility curve. The automatically recorded curve is obtained in less than 10 minutes.

In a previous study, osmotic haemolysis was cinematographically recorded under a phase-contrast microscope by gradually decreasing the salt concentration of the medium surrounding the erythrocytes. Gradual decrease of ionic strength in the suspending medium, while maintaining a practically constant *pH*, was achieved by surrounding the drop of suspended erythrocytes with a mixed ion exchanger (Danon, 1961).

It was found that after a few minutes, during which the cells changed from bidiscoidal erythrocytes to cap form and then became spheres, about 10 to 20% of the cells released their haemoglobin and became 'ghosts'. Subsequently, the majority of the cells followed each other very closely in haemolyzing, and very often a few cells were seen to haemolyze at the same time. A small number of cells (about 2%) remained spherical and resisted haemolysis for a few seconds after the rest were all ghosts. Finally, these cells also released their haemoglobin and the whole microscopic field was covered with ghosts visible as fine circles. The inside of the ghosts had the same colour as the surrounding medium, indicating the same concentration of haemoglobin. It was suggested that osmotic haemolysis obtained by gradually decreasing the ionic strength of the surrounding medium observed under the microscope could serve as the basis for a new fragility

test. What is actually observed under the microscope is a sequence of events, each taking place in a medium of ionic strength lower than the previous one.

In the present paper a new fragility test is described in which a single volume of 0.075 ml. of a 1 to 1 in 10 dilution of blood in isotonic NaCl solution is used. The curve of degree of haemolysis versus ionic strength is automatically recorded. Reduction of ionic strength is achieved by dialysing the given volume of blood suspension in isotonic saline against distilled water. The measurement of degree of haemolysis is based on the increasing transparency of the erythrocyte suspension while haemolysis takes place. Recording this increasing light transmission as a function of time provides the same information as recording the degree of haemolysis as a function of decreasing salt concentration, after the curve of decrease of salt concentration as a function of time is established. The fragility curve is obtained in less than 10 minutes.

APPARATUS

The apparatus is illustrated in Figure 1.

A rectangular cell, two walls of which are made of a dialysing membrane, serves as a container for the blood suspension. A test tube of 11.5 mm. internal diameter containing 3 ml. of distilled water is placed in an instrument, which is essentially a colorimeter with a recorder,

S.T. = stabilizer. L.S. = light source. H.F. = heat-absorbing filter. L. = lenses. G.F. = green filter (500 m μ). D.W. = distilled water. T.T. = test tube. C.C. = container cell. B.S. = blood suspension. ThB. = thermostatic bath. B.T. = black tube. P.C. = photoelectric cell. Diff. C. = differentiating circuit. C.F. = cathode follower. REC. = recorder.

between the source of light (using a 500 m μ filter) and the photoelectric cell. The frame with the cell containing the blood suspension is introduced into the distilled water so that the dialysis membrane windows are perpendicular to the beam of light. A special device ensures that the cell can be placed only in its correct position. The temperature of the test tube of distilled water can be kept constant.¹ A mechanical-electronic device permits a simple recording of the sigmoid fragility curve or, alternatively, its derivative (Ponder, 1948; Bolton, 1949) to be obtained as desired. The design of the instrument is described elsewhere (Danon, Frei, Frei, and Lipkin, 1963).

Recording starts as soon as the container cell has been introduced into the distilled water.

THE CONTAINER CELL A frame is made of stainless steel 0.6 mm. thick, with a rectangular cross section as shown in Figure 2. The elongated hole is 5 mm. wide and 25 mm. long, thus delimiting a volume of 0.075 ml. for the blood suspension. A piece of dialyser tubing² is mounted on this frame and its lower end cemented with Plicene³ cement or Apiezon⁴ sealing wax W. The membrane is mounted humid and cut 1 mm. below the lower end of the frame. It is then dipped up to the window in acetone for a few seconds to dehydrate the lower end of the membrane. After evaporation of the acetone the lower open end of the membrane is sealed with the help of a soldering iron⁵.

It is important that the tube is well immersed in saline before mounting, so that it becomes malleable and slightly stretches on the frame. After the membrane is mounted it is blotted on the outside. If water can be seen inside it

FIG. 2. The test container cell. On the stainless steel frame 0.6 mm. thick and 11.3 mm. wide is mounted the dialysis membrane (DM). The lower end of the membrane is cemented to the frame by Plicene cement (P), so that the suspension of blood introduced into the container cell (CC) does not leak out, and the distilled water does not leak in. The top transversal part of the frame serves for the correct positioning of the container cell.

is blotted with a strip of filter paper. As the dialysis membrane is stretched on the frame, the distance between the membrane is determined by the thickness of the frame, i.e., 0.6 mm., in this case. The dialysis membrane must be humid but not wet before the blood suspension is introduced. This will avoid the appearance of waves on the cellophane membrane when the blood suspension is introduced, which might disturb the regularity of the blood suspension layer.

METHOD

A heparinized capillary tube, 75 mm. long and 1.2 to 1.4 mm. in diameter, of the type used for the microhaematocrit, is mounted in a Pasteur pipette as described by Dacie (1956) for fixed volume measurements. A simpler alternative is to mount the capillary in a polythene⁶ pipette, which does not need sealing, as the tip hole of the pipette happens to be of correct diameter. The capillary is filled from the finger tip or from a test

¹The first experiments were performed using a simple colorimeter. However, it was found that accurate reading every 20 or 30 seconds (in order to obtain a curve composed of at least 20 points) is a difficult task, while automatic recording gives a much more detailed curve, marking a point every two seconds. Moreover, the simple colorimeter gave somewhat erratic results due to the irreproducibility of the container cell position. The simple colorimeter may still be a useful tool for a first rapid screening of osmotic fragility.

²Arthur H. Thomas Co., Philadelphia 5, Pa., U.S.A.

³Cenco No. 1144.

⁴Distributed by Shell Companies.

⁵A simple alternative suggested by Dr. Yael Frei consists of knotting the dialysis tube below the frame.

⁶X-Lon Products Ltd., 48 Gillingham Street, London, S.W.1. (catalogue No. TO20).

tube containing heparinized blood, and its content, about 0.05 ml., is emptied into a 5 ml. beaker, containing 0.5 ml. of saline buffered to pH 7.4. The solution is prepared by dissolving 8.5 g. of analytical grade NaCl in 960 ml. of glass-distilled water and adding to it 40 ml. of buffer composed of 24 ml. Na veronal M/10 and 16 ml. HCl N/10.

The suspension of diluted blood spread on the relatively large bottom of the beaker is aerated as recommended by Creed (1938), by circular stirring of the beaker, and then introduced into the container cell with the help of a 1 ml. syringe, with a bent needle, number 22. The needle is inserted between the frame and the membrane down to the base of the container cell. As the blood fills the cell from the bottom, the air leaves the cell through the opening formed by the lifted membrane. It is important that the suspension fill the cell to the top. Air bubbles must be avoided. This can be easily achieved by using a siliconized syringe and a siliconized needle. The cell containing the blood suspension is blotted from outside, and is now introduced into the test tube of distilled water situated between the source of light and the photoelectric cell. The recording is finished when the plateau indicating completion of haemolysis is reached (about seven minutes for normal human blood).

RESULTS

An osmotic fragility curve of normal adult human blood, obtained by the above method at room temperature, is shown in Figure 3.

Figure 4 represents the reproducibility of the fragility curve when samples of the same blood were tested seven times consecutively. A new dialysis membrane was mounted on the frame for every test. A similar experiment in which the dialysis membrane was not changed, but the same mounted membrane was rinsed with isotonic NaCl solution after every test and then the container cell refilled with blood suspension, showed a slightly better reproducibility if the first test was ignored. The difference of a few seconds in the time required for haemolysis to take place between the first and the following tests, using the same membrane, is prob-

FIG. 4. Reproducibility of the fragility curve. Seven different curves of the same blood plotted on the recorder paper. The first curve obtained (1) indicates a slight delay in haemolysis. It does not occur if the instrument is left to reach equilibrium with room temperature for 20 minutes before starting.

ably due to a slower rate of diffusion through the new membrane. Seven osmotic fragility curves obtained from the same blood by a conventional method, (Dacie, 1956) are plotted in Fig. 5 on the recorder paper for comparison. Figure 6 represents the fragility curves recorded by the newly described method of blood of a 2-day-old child, of red cells spherized by the process of heating them to 48°C. and then cooling back again to 37°C. (Carlsen and Comroe, 1960), and of three patients with abnormal osmotic red cell fragility.

DISCUSSION

The principal difference between the present method and the conventional osmotic fragility test is in the manner by which the cells are subjected to hypotonic solutions. In the conventional test, the cells are subjected to a sudden or drastic decrease in the ionic strength of the suspending medium. In the present test, haemolysis is obtained by continuous decrease of the salt concentration of the suspending medium.

FIG. 3. An automatically recorded osmotic fragility curve of a normal human blood. A Rustrak recorder marking a point every two seconds, advancing at a speed of two vertical lines per minute, was used.

FIG. 5

FIG. 5. *Reproducibility of a conventional osmotic fragility test. Same as Fig. 4 but the curves were obtained by a conventional method (Dacie, 1956). They were plotted on recorder paper to facilitate comparison with Figure 4.*

FIG. 6. *Curves of abnormal osmotic fragility. The dotted area represents the range of normal fragility of human erythrocytes in the present experimental conditions.*

FIG. 6

The fact that red blood cell ghosts can be obtained by dialysing a suspension of erythrocytes against hypotonic solution or distilled water has been previously established (Danon, Nevo, and Marikovsky, 1956). It was found that this method of haemolysis brings about the release of haemoglobin at a slightly lower ionic strength than the method of putting the cells directly into hypotonic solutions. It was also found that this difference is negligible if the erythrocytes are not washed, and if haemolysis takes place in the presence of diluted plasma (Katchalsky, Kedem, Klibansky, and De Vries, 1960). These are the experimental conditions in the present fragility test.

The curves obtained by the present method describe the degree of haemolysis on the ordinate as a function of time on the abscissa. Salt concentration in a dialysis bag decreases exponentially when dialysed against distilled water. A curve was therefore established that enables the concentration of NaCl to be determined at various points on the abscissa, so that the results of this test may be compared with conventional osmotic fragility tests.

The apparatus was set up as usual, but after one minute the container cell was taken out of the test

tube of distilled water, the external water blotted, and 0.05 ml. of the internal blood suspension was taken out and introduced into a vessel of an Aminco-Cotlove chloride titrator.⁷ This procedure was repeated seven to 10 times, each time starting a new fragility test of the same blood in the same mounted membrane that had been rinsed in saline, and allowing dialysis to take place each time for one more minute. In Figure 7 the NaCl concentration (calculated from the chloride titration) is plotted as a function of time.

Determination of the salt concentration at which the various points of the curve were marked is only necessary when data obtained by the present method are to be compared with other results that have been obtained by conventional methods. Normally a comparison is made with the curves obtained with the range of normal blood determined by using controls. This procedure is always desirable when blood is tested for osmotic fragility (Wintrobe, 1961).

To determine whether the cells near the internal surface of the dialysis membrane are subjected to exaggerated hypotonicity, *i.e.*, whether the ionic

⁷American Instrument Co., Silver Springs, Maryland.

FIG. 7. NaCl concentration, inside the container cell, on the ordinate as a function of time on the abscissa. The curve was obtained by titrating chloride after various times of dialysis.

strength inside the recipient cell is practically uniform, it is argued that the salt concentration in the container cell will be uniform during the whole experiment if mixing inside the container (ions in free solution) is much faster than the permeability of the salt through the membrane. As any change of concentration near the inner surface of the membrane must bring about convection currents in addition to diffusion, this may be safely assumed. If the container cell with the blood suspension is introduced into a test tube of distilled water, vertically placed, in front of a binocular microscope in the horizontal position, the blood cells near the internal surface of the dialysis membrane can be seen to climb as dialysis progresses. Furthermore, in a well-stirred compartment limited by dialysis membranes, the rate of decreasing tonicity is proportional to the number of ions leaving through the membrane per unit of time and inversely proportional to the whole volume of internal solution. In the fragility test described above, this should be illustrated by haemolysis occurring in a shorter time when the

distance between the membranes is diminished; in other words, if the frame is thinner. The results presented in Fig. 8 confirm this assumption.

In view of these findings it is suggested that under the experimental conditions described, the curve obtained by this new method may be considered as an osmotic fragility curve ('fragiligram') in the classical sense. It presents the following advantages:—It requires minute amounts of blood. It uses a single volume, which avoids errors in measurement of the blood volume and hypotonic NaCl solution volumes. It is rapid (six to 10 minutes). It can be directly recorded. The curve contains more information than the one obtained from a test made in 20 test tubes. It does not require a skilled technician. The possibility of repeating an osmotic fragility test sometimes using the same dialysing membrane, and the fact that only minute amounts of blood are required to repeat the test several times, permits a control to precede or follow every abnormal result using identical experimental conditions.

FIG. 8. A comparison of the time required for haemolysis to take place in two container cells of different thickness. It can be seen that in the cell in which the distance between the two membranes is of 0.35 mm., i.e., of approximately half the volume of the usual container cell, every stage of the haemolysis takes place at slightly more than half the time it takes to occur in the usual cell in which the distance between the membranes is 0.6 mm.

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