

THE APPEARANCE OF ERYTHROCYTE MEMBRANE ELEVATIONS

Effects of cooling rates.

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ABSTRACT

Low cooling rates during the freezing procedure of normal human blood reveals red cell membrane elevations in freeze-etch electron microscopy.

When high cooling rate is applied, these morphological changes are present, if the blood samples are quenched from 5°C. The number of elevations is strongly reduced by low pH and glycerol. Elevations are not observed in ghosts. The formation of intramembrane particle aggregation is differently affected by many conditions.

INTRODUCTION

Freeze-etch electron microscopy of normal human erythrocytes reveals membrane elevations at pH 7.4 (6). We previously reported that these elevations are reduced in number at acidemic and alkalemic conditions (7), and that the use of glycerol as cryoprotectant resulted in the absence of these elevations (6).

In the present study we have investigated the influence of the cooling rate on the appearance of elevations, and of the temperature at which the blood sample is quenched into the cooling medium.

We further studied the effects of pH, different anticoagulant agents, and glycerol as cryoprotectant on the temperature dependent appearance of membrane elevations. The aggregation of intramembrane particles (IMP) under all these conditions was examined too.

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MATERIALS AND METHODS

Peripheral venous blood of healthy volunteers was obtained by venapuncture. NH_4 -Heparin, and Acid Citrate Dextrose (ACD) at pH 7.4, and at pH 4.5 were used as anticoagulant. Ghosts were prepared according to the method of Dodge et al. (2).

For freeze-fracturing small drops of differently treated blood-samples were transferred into freeze-etch specimen holders, i.e. silver cylinders with a diameter of 1 mm and a height of 3 mm (13), and worked up in a Polaron machine. Three cooling procedures were used: 1. A low cooling rate, in which the specimen holders were brought to -20°C , kept at that temperature for 30' and subsequently quenched into solid/liquid nitrogen. 2. An intermediate cooling rate: Specimen holders were quenched into solid/liquid nitrogen (11). 3. A high cooling rate: Specimen holders were quenched into solid/liquid Freon 22 and thereafter in solid/liquid nitrogen. Cryoprotected samples were treated with glycerol to a final concentration of 30%.

Only intact replicas with a diameter equal to the diameter of the specimen holder were used for quantitative evaluation. The percentage of erythrocyte fracture faces with elevations is called the elevation value (e-value) of a given area.

RESULTS

When venous heparinized blood was cooled at intermediate rate, local differences in e-value within one replica were observed.

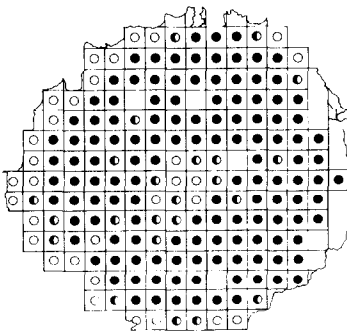


Fig. 1. A coppergrid with a replica from venous heparinized blood cooled at intermediate rate.

Each square represents a hole in the grid. The spots in the squares represent global e-values (see text). Empty squares could not be counted.

A three partition can be seen from fig. 1: 1) At the periphery and the centre of the replica (□ squares) e-values lower than 30% are found. The number of fracture faces counted in these areas is 252. 2) The (◐) squares represent areas in which e-values of 30-60% are found. The number of fracture faces counted here is 237. 3) The (◑) squares represent areas in the replica with e-values of 60% or higher. The number of fracture faces counted is 1358.

Cooling at high rate from 23°C and at pH 7.4, resulted in frac-

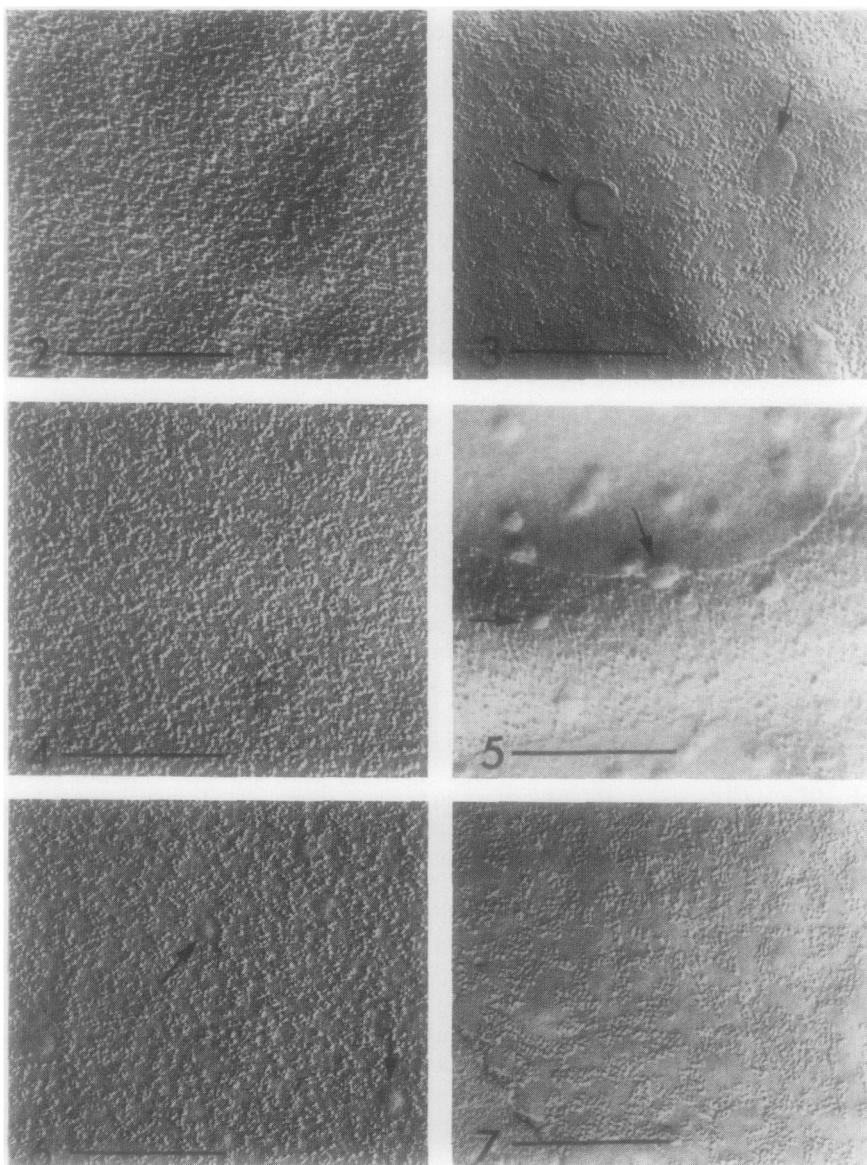


Fig. 2-6: Presence or absence of elevations (marked by arrows) and IMP aggregation in intact red cells at different cooling rates and conditions: 2) High rate cooling, all conditions. 3) Low rate cooling, and heparin. 4) Low rate cooling, and ACD pH 4.5. 5) Intermediate rate cooling, and heparin or ACD pH 7.4. 6) Low rate cooling, heparin and glycerol.

Fig. 7: Presence of IMP aggregation and absence of elevations in ghosts, cooled at low rate.
The bars represent 0.3 μ m.

ture faces without elevations. At low rate cooling at pH 7.4 fracture faces with elevations and comparable e-values were found throughout the whole replica.

From these results one could deduce that the temperature of quenching may be of importance. Therefore the samples were cooled to 5°C, kept at that temperature for 3' and subsequently quenched in solid/liquid Freon 22, according to the high rate cooling procedure. Elevations and a slight aggregation of IMP's were found. To test whether these temperature dependent membrane changes are reversible, the samples were first cooled to 5°C, kept there for 3', subsequently kept at 23°C for 3', and immediately thereafter quenched according to the high rate cooling procedure. No elevations and IMP aggregation were observed, as after cooling at high rate directly from 23°C. The same effect was observed after quenching with uncooled tweezers in the preceding experiment after cooling to 5°C.

The effects of pH, anticoagulant agents and glycerol on the appearance of elevations and IMP aggregation were studied at the three cooling rates described in Materials and Methods. At high rate cooling no elevations and IMP aggregation could be detected, under all experimental conditions (fig. 2). At low rate cooling the formation of both elevations and IMP aggregation was most obvious if heparin was used as anticoagulant agent (fig. 3). The use of ACD at pH 7.4 instead of heparin reduced the IMP aggregation to a certain extent, but had no reducing effect on the presence of elevations. The reduction of the pH of ACD to pH 4.5 did not change this slight aggregation of IMP's, but resulted in the absence of elevations (fig. 4). Using intermediate rate cooling the erythrocytes showed elevations with heparin or ACD at pH 7.4. However, IMP aggregation was not observed (fig. 5). ACD at pH 4.5 resulted in the absence of elevations.

In the presence of 30% glycerol onsets of elevations and a slight IMP aggregation were only observed in heparinized blood at the low cooling rate (fig. 6).

Elevations could never be found in ghosts under all conditions described. When ghosts were cooled at low rate without glycerol more or less the same IMP aggregation was found as in intact red cells (fig. 7).

DISCUSSION

Differences in cooling rates have been observed within a freeze-etch specimen holder (12). In the center and the periphery the highest rates are observed. If the local differences found in our experiments at intermediate cooling rate are caused by this temperature effect, the presence of more fracture faces without elevations can be expected in the whole replica after cooling at high rate. The use of solid/liquid Freon 22 has been reported to result in an approximately three times higher cooling rate than obtained by the use of solid/liquid nitrogen (1), and resulted in our experiments in the absence of elevations in the replica. In contrast,

no local differences were found in the presence of elevations after cooling at low rate. From these results one may conclude that the presence of elevations is primarily dependent on "low" cooling rates. Further investigations at high rate cooling showed that the temperature of quenching plays a role in the appearance of elevations, as no elevations are present after quenching at 23°C, and the elevations appear after quenching at 5°C. This temperature dependent appearance of elevations at 5°C is reversible.

In comparing the circumstances of the formation of elevations and IMP aggregation we observed one similarity: Low rate cooling is a condition of the presence of both membrane changes. However, the occurrence of elevations is not linked to IMP aggregation, as IMP aggregation is absent at intermediate rate cooling, whereas elevations are present. In this respect it may be noted that strong particle aggregation was found in spectrin-actin free ghosts (5), which was explained to be the result of changes in the physicochemical state of the membrane lipids. At low pH the elevations disappear, while IMP aggregation is still present. This low cooling rate dependent IMP aggregation may be related with that found in spectrin-actin depleted (3) and spectrin-actin free (5) ghosts.

The mechanism of the appearance of elevations on the erythrocyte fracture faces is still hardly understood. From our results by quenching from different temperatures (23°C and 5°C) it is clear that it is not simply a freezing artefact due to the formation of ice crystals or locally raised concentrations of salt during freezing. It may be possible that other temperature dependent molecular rearrangements may occur between 23°C and 5°C. In this respect other spontaneous membrane changes may be of interest. Such changes have been observed at 4°C. Some glycoproteins in intact human red cells show a difference in availability to a probe at 37°C and at 4°C. pH Dependent variation in availability was observed too, and after ghost preparation no changes could be detected (8). Spontaneous vesiculation of sheep erythrocyte ghosts occurs at 4°C (9). The appearance of elevations in human erythrocytes may be caused by a similar mechanism. A release of spectrin free vesicles is observed in intact human red cells after ATP depletion (10) and it has been suggested that the spectrin meshwork plays a role in the phenomenon of exocytosis (4). Research in this direction will be carried out.

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REFERENCES

1. Costello, M.J. and Corless, J.M. (1978) The direct measurement of temperature changes within freeze-fracture specimens during rapid quenching in liquid coolants. *Journal of Microscopy* 112, 17-37.

2. Dodge, J.T., Mitchell, C. and Hanahan, D.J. (1963) The Preparation and Chemical Characteristics of Hemoglobin - free Ghosts of Human Erythrocytes. *Archives of Biochemistry and Biophysics* 100, 119-130.
3. Elgsaeter, A.J. and Branton, D.J. (1974) Intramembrane particle aggregation in erythrocyte ghosts I. The effects of protein removal. *Journal of Cell Biology* 63, 1018-1030.
4. Elgsaeter, A.J., Shotton, D.M. and Branton, D.J. (1976) Intramembrane particle aggregation in erythrocyte ghosts II. The influence of spectrin aggregation. *Biochimica Biophysica Acta* 426, 101-122.
5. Gerritsen, W.J., Verkleij, A.J. and Van Deenen, L.L.M. The lateral distribution of intramembrane particles in the erythrocyte membrane and recombinant vesicles. *Biochimica Biophysica Acta*. In press.
6. Goekoop, J.G., Spies, F., Bierman-Van Steeg, C., Vrielink, R., Van Kempen, G.M.J. and De Vries, E. (1978) pH Dependent behaviour of erythrocyte membrane elevations. *Cell Biology International Reports* 2, 139-145.
7. Goekoop, J.G., Spies, F., Wisse, D.M., Vrielink, R., De Vries, E. and Van Kempen, G.M.J. (1978) pH Dependent behaviour of erythrocyte membrane elevations II. Occurrence in moderate acidemic and alkalemic conditions. *Cell Biology International Reports* 2, 397-402.
8. Luthra, M.G., Friedman, J.M. and Sears, D.A. (1978) Effects of pH and Temperature on the Interaction of an Impermeant Probe with Surface Proteins of the Human Red Blood Cell. *Journal of Biological Chemistry* 253, 5647-5653.
9. Lutz, H.U., Barber, R. and McGuire, R.F. (1976) Glycoprotein enriched Vesicles from Sheep Erythrocyte Ghosts obtained by Spontaneous Vesiculation. *Journal of Biological Chemistry* 251, 3500-3510.
10. Lutz, H.U., Liu, S. and Palek, J. (1977) Release of spectrin-free vesicles from human erythrocytes during ATP depletion. *Journal of Cell Biology* 73, 548-560.
11. Umrath, W. (1974) Cooling bath for rapid freezing in electron microscopy. *Journal of Microscopy* 101, 103-105.
12. Van Venrooij, G.E.P.M., Aertsen, A.M.H.J., Hax, W.M.A., Ververgaert, P.H.J.T., Verhoeven, J.J. and Van Der Vorst, H.A. (1975) Freeze-etching: Freezing velocity and crystal size at different locations in samples. *Cryobiology* 12, 46-62.
13. Ververgaert, P.H.J.T. (1973) Ultrastructural analysis of model membranes. Ph.D. Thesis. Utrecht, pp. 28-29.