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# Hemolysis and Morphological Changes of Erythrocytes with Freezing\*

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## Abstract

In order to clarify the mechanism of hemolysis resulting from freezing and thawing, the morphological changes of erythrocytes in frozen blood were compared with the amount of hemolysis after thawing. Specimens of rabbit blood were prepared by the freeze-drying method after having been frozen at various temperatures and were then observed with optical and electron microscopes. Most of cells frozen at high temperatures appeared shrunken and without intracellular ice, while cells frozen at low temperatures retained their original shapes but had many small cavities, presumably formed by intracellular ice crystals. The mechanism of hemolysis in blood, frozen at various temperatures, was interpreted from this morphological point of view.

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## 1. Introduction

Numerous experiments have been reported on successful freeze-drying of microorganisms and viruses, since the method was first used to preserve living cells about half a century ago. However, in spite of many attempts, little progress has been made in obtaining viable mammalian cells after freeze-drying. With the exception of MERYMAN<sup>1)</sup>, many investigators have attempted to freeze-dry erythrocytes, but from the results reported, they were unable to obtain recovery after rehydrating the dried specimen. NEI and his collaborators<sup>2)</sup> have attempted to freeze-dry blood cells after very rapid freezing, but were unable to obtain recovery of intact erythrocytes after rehydration.

Because of the difficulties encountered in freeze-drying mammalian cells,

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a thorough investigation of cell damage during freezing, drying and rehydration is essential.

RAPATZ and LUYET<sup>3)</sup> have shown, by electron microscopy, that when frog's blood is frozen at cooling velocities of several hundred degrees per second, intracellular ice is formed both in the nucleus and in the cytoplasm, whereas, at lower cooling rates, only extracellular freezing occurs. Recently RAPATZ and LUYET<sup>4)</sup> reported a correlation between hemolysis and the presence of intracellular ice. Using normal and glycerolated bovine blood, they found that rapid freezing (by immersing thin films into a bath at  $-80^{\circ}\text{C}$ ) resulted in maximum hemolysis and the formation of intracellular ice, while freezing at higher temperatures ( $-20^{\circ}\text{C}$  with glycerolated blood or  $-40^{\circ}\text{C}$  without glycerol) caused minimal hemolysis and only extracellular ice formation.

The work reported here is the first of a series of experiments undertaken in our laboratory. Morphological changes of rabbit red cells subjected to freezing were observed and correlated with hemolysis after freeze-thawing. The effect of rates of cooling on cell morphology and cell damage was also investigated.

## II. Materials and Methods

Rabbit whole blood, containing potassium oxalate to prevent coagulation, was used throughout this study. Twenty  $\text{mm}^3$  of blood were placed between a cover glass  $150\ \mu$  thick and  $2 \times 3\ \text{cm}$  on edge, and a sheet of aluminum foil the same size and  $25\ \mu$  thick. To insure a uniform thickness of the layer of blood, the preparation was pressed between two microscope slides. The specimen thus prepared was frozen by abrupt immersion into an isopentane bath maintained at either  $-10^{\circ}$ ,  $-20^{\circ}$ ,  $-30^{\circ}$ ,  $-40^{\circ}$ ,  $-50^{\circ}$ ,  $-80^{\circ}$ ,  $-150^{\circ}\text{C}$  or a liquid nitrogen bath. After 5 minutes the aluminum foil was pulled from the glass to which the frozen blood tightly adhered. The blood was thawed by immersing the cover glass in 5 ml of physiological saline at  $20^{\circ}\text{C}$ . The extent of hemolysis resulting from freeze-thawing was determined as follows: The hemoglobin in the supernatant fluid of the treated blood was converted to cyanmethemoglobin and the color density measured with an electrophotometer. Morphological observations of the frozen cells were made on freeze-dried specimens. To prepare such specimens the cover glass with the frozen blood adhering to it was transferred to a container previously cooled to  $-30^{\circ}$  or  $-50^{\circ}$  or  $-70^{\circ}\text{C}$ . Freeze-drying was carried out at these temperatures for 8 hours under a vacuum of  $10^{-3}\ \text{mmHg}$ , followed by further drying for 6 hours at  $10^{-5}\ \text{mmHg}$ . The dried preparation while still on the cover glass was observed as a whole with the light microscope at low magnification. Cellular

detail was studied at higher magnification after the cells were suspended in cedar oil. For electron microscopy the freeze-dried specimen was fixed in 2% osmium tetroxide-alcohol and embedded in a mixture of 1 part methyl and 9 parts n-butyl methacrylate. Ultra-thin-sectioned specimens were observed with JEM 4C electron microscope under an accelerating voltage of 50 KV.

### III. Results

#### *Hemolysis by freezing*

The extent of hemolysis of erythrocytes, frozen at different temperatures and thawed at 20°C, is given in Fig. 1. Freezing at relatively high temperatures

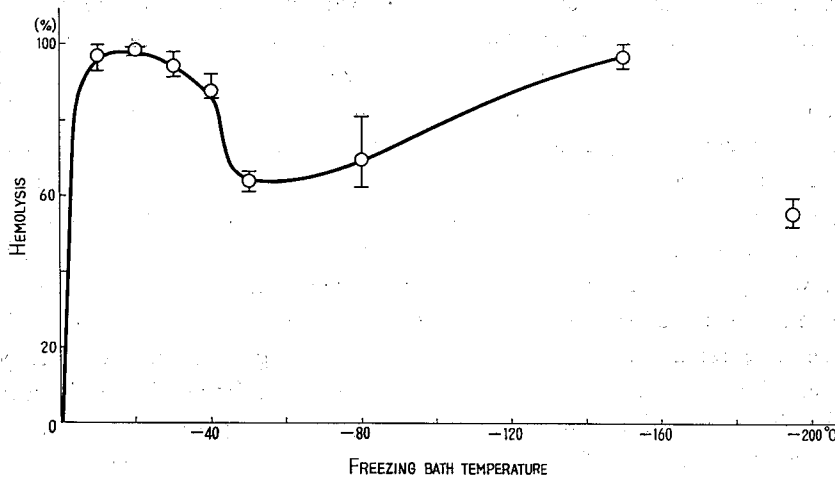


Fig. 1. Hemolysis curve in rabbit blood frozen at various temperatures

such as  $-10^{\circ}$  or  $-20^{\circ}\text{C}$  and at the extremely low temperature of  $-150^{\circ}\text{C}$  resulted in complete hemolysis, whereas freezing at  $-50^{\circ}\text{C}$  gave the lowest value, about 60%. Hemolysis in the case of direct immersion in liquid nitrogen gave similar values to those obtained at  $-50^{\circ}\text{C}$ .

#### *Morphology of frozen blood*

Observations with the light microscope showed most of those cells frozen at high temperatures to be shrunken, similar to collapsed balloons (Figs. 4 a and 4 b), while the cells frozen at lower temperatures maintained their usual shape, with evidence of some intracellular granularity (Figs. 5 a and 5 b).

Electron microscopic study showed the following predominant cell changes: Cells frozen at  $-30^{\circ}\text{C}$  were irregular in shape and shrunken, but were uniform in density just as the controls (Figs. 6 and 7). There was no evidence of

intracellular ice. In preparations frozen at  $-50^{\circ}\text{C}$  two types of cells were noted; one similar to those frozen at  $-30^{\circ}\text{C}$  and the others containing numerous small cavities, presumably left after the sublimation of ice crystals (Fig. 8). Cells frozen at  $-150^{\circ}\text{C}$  contained the same type of cavities, however, they were much more numerous and smaller as compared to those formed at  $-50^{\circ}\text{C}$  (Fig. 9). Cells frozen in liquid nitrogen were similar to those frozen at  $-50^{\circ}\text{C}$ .

#### IV. Discussion

The results indicate that hemolysis from freezing is dependent upon the temperatures (cooling velocities) to which the blood is subjected. In blood frozen at  $-50^{\circ}\text{C}$  about 60% of the cells were hemolyzed, whereas the cells in blood frozen at higher or lower temperatures were completely hemolyzed.

The morphological observations of frozen blood helps to interpret the mechanism of cell damage during freeze-thawing. Complete hemolysis of cells in blood frozen at high temperatures (about  $-20^{\circ}\text{C}$ ) which are devoid of intracellular ice can be explained by salt injury as suggested by LOVELOCK<sup>5</sup>. Complete hemolysis of the blood frozen at  $-150^{\circ}\text{C}$  is most probably the result of mechanical damage caused by intracellular freezing. The existence of frozen (as shown by intracellular cavities) and unfrozen cells in the same preparation immersed at  $-50^{\circ}\text{C}$ , suggests that some of the unfrozen cells avoid salt injury because they remain in the critical temperature range for only a short period of time. The reduction in hemolysis of the blood frozen in liquid nitrogen may be the result of a lower rate of cooling, as compared to that frozen in isopentane bath at  $-150^{\circ}\text{C}$ , however additional evidence is needed to clarify these results.

Although the experimental conditions, *i. e.*, species of animal, from which

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**Figs. 2 to 5.** Optical micrographs.  $\times 2,000$

**Fig. 2.** Undried control in the liquid state

**Fig. 3.** Blood smear air-dried at room temperature

**Figs. 4 a and 4 b.** Frozen and dried at  $-20^{\circ}\text{C}$ . Shrunken cells

**Figs. 5 a and 5 b.** Frozen in liquid nitrogen and dried at  $-50^{\circ}\text{C}$ .  
Normally shaped but granulated cells

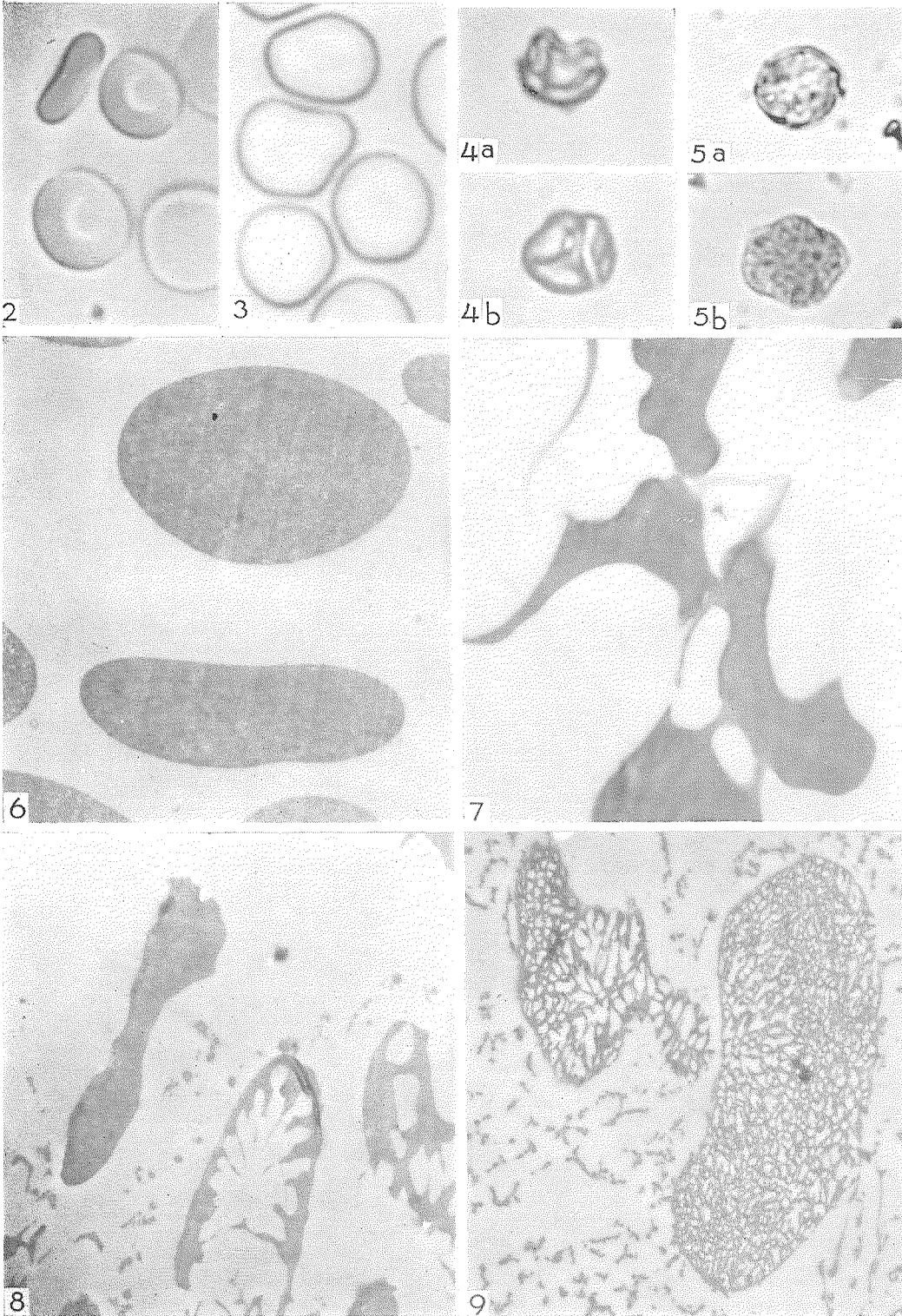
**Figs. 6 to 9.** Electron micrographs of thin-sectioned specimens.  $\times 10,000$

**Fig. 6.** Unfrozen control

**Fig. 7.** Frozen and dried at  $-30^{\circ}\text{C}$ . Shrunken cells

**Fig. 8.** Frozen and dried at  $-50^{\circ}\text{C}$ . Two different cell types

**Fig. 9.** Froen at  $-150^{\circ}\text{C}$  and dried at  $-70^{\circ}\text{C}$ . Note many small  
intracellular cavities



the blood was collected, and the method and rate of cooling, were different in the work of RAPATZ and LUYET<sup>4)</sup> and ours, the general trend of the results is, in certain ranges of freezing temperatures, quite similar in both experiments; an increase in cooling velocity results in an increase in morphological and functional injury of the cells.

More precise and detailed observations as well as more quantitative measurements should be made to elucidate the mechanism of cell injury in freezing of blood. Further investigations are therefore still in progress.

The present work was reported as an additional comment to RAPATZ' paper at XIth International Congress of Refrigeration in Munich in 1963.

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