Rapid Freezing and Thawing of Whole Blood. (22106)

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Destruction of erythrocytes following freezing and thawing is so familiar a phenomenon as to have become a recognized procedure for obtaining complete red cell hemolysis. Only in very recent years have the basic principles underlying freezing in biology been sufficiently well established to suggest some of the causes of this hemolysis as well as means by which it might be avoided. It has long been recognized that there is a substantial difference between the results of rapid and slow freezing in biology. More recently this has been placed on a less empirical basis by the demonstration that slow freezing causes the formation of exclusively extracellular ice crystals which plasmolyze but do not necessarily rupture the cells while rapid freezing causes the formation of numerous intracellular ice crystals(1). When blood is slowly frozen, it has been shown that it is the dehydration and resultant electrolyte concentration which destroys the cell rather than the physical presence of the extracellular ice crystals(2). The success of glycerine in preventing the hemolysis of frozen erythrocytes can be attributed to the reduction in dehydration effected by the ability of glycerine to bind to itself and retain in solution much of the water which would otherwise have crystallized(3). Rapid freezing entails a considerably different problem and the only hope for subsequent survival lies in obtaining a crystal size sufficiently small to be benign despite the intracellular distribution. Luyet(4) has recovered morphologically intact erythrocytes after rapidly freezing and thawing a thin smear of blood on a cover slip but there are no other reports known to the authors in which the surface-to-volume limitations for rapid heat exchange were adequately recognized. Where the formation of extremely small intracellular crystals is tolerated it is presumed that the denaturing effect of removing water from solution can be reduced to an insignificant level by the rapid attainment of and storage at low temperature.

Since both the attainment of small ice crystals and the prevention of denaturation depend upon very rapid freezing to low temperature, the major problem is simply one of efficient heat exchange. Studies in the kinetics of freezing and thawing(5) have shown that the most efficient shape for heat exchange is the very small sphere and that the exchange of latent heat, particularly in thawing, is so demanding that every possible advantage that may be gained through specimen geometry must be exploited to the utmost if the transition from liquid to solid and back to liquid is to be sufficiently rapid. Further discussion of the theory and mechanism of the freezing of whole blood may be found in a more extensive paper on this subject to be published elsewhere(6).

Method. In view of the above remarks it can be seen that the first obstacle to successful rapid freezing of whole blood is the attainment of sufficiently fine droplet form without injury. Many means have been tried: the conventional atomizer, spinning disc and many types of jet, all with widely varying degrees of success. The method ultimately adopted was to spray the blood through a rapidly oscillating capillary jet drawn to an 0.18 mm aperture from 2.5 mm I.D. vinyl tubing. The fine droplets, ranging from .45 to .9 mm in diameter, are sprayed directly onto the surface of liquid nitrogen or liquid air where they float for a second, then sink to the bottom of the vessel. Reconstitution is achieved simply by sprinkling the frozen blood, which now resembles a fine sand, into plasma or physiological saline at 42°C. Throughout the developmental stages, spontaneous hemolysis was used as the criterion for erythrocyte injury.

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TABLE I. Post-Transfusion Survival of Erythrocytes after Freezing and Thawing Expressed as Percent of Whole Blood Radioactivity 15 Min. after Injection.

Subject	Days post-transfusion ¼	1	2	3	4	5	6	7	8	12	13	20	31	37	44	56	62
HM (autog.)	94	86	82	77	87		70	54			55	45	32	35	21	15	12
EK (homol.)	101	82	78	67	69	64			52	55		01					

Results. Following the standardization of the jet method as the least traumatic, recovery of intact erythrocytes was still rarely better than 50%. Because of these consistently poor although promising results, a compromise was introduced in the form of added dextrose designed to reduce the crystal growth rate(6) as well as to bind additional water and reduce the ultimate amount of ice formation. This produced an immediate improvement in percent recovery when added at the rate of 0.07 g/cc whole blood, reducing the spontaneous hemolysis to between 15 and 20%.

At this stage, 100 ml of dog blood were frozen, thawed, tagged with Cr^{51}(7) and reinfused into 2 dogs in order to determine the *in vivo* survival of the erythrocytes. Partly because of the fragility of dog erythrocytes and partly because of poor blood handling technic in this particular experiment, over 60% spontaneous hemolysis resulted from freezing and thawing alone. Of those cells remaining intact, only 18% were still present in the recipient circulation after 24 hours.

Following this equivocal result, every step of the procedure was reexamined. No major changes were made, but many minor improvements such as a change from polyethylene to vinyl tubing, covering the tubing with a rubber coat where it passed under the rollers of the peristaltic pump, making tubing and jet one piece instead of replaceable through a Luer-lok joint, and increasing the efficiency of heat exchange in the thawing bath, all contrived to reduce the damage to freshly drawn, citrated blood with added dextrose to consistently less than 3% spontaneous hemolysis after freezing and thawing. When dextrose was not added, 10 to 15% hemolysis could be expected. Inasmuch as another dog transfusion, whether positive or negative, would have to be confirmed in man, this intermediate step was bypassed and a reinfusion of Cr^{51} tagged blood was made in a human volunteer.

Two 100 ml aliquots of blood were drawn, each into a solution containing 0.32 g sodium citrate, 0.1 g citric acid and 7.3 g dextrose in 25 ml of solution. One of these contained, in addition, 1.0 μc of Cr^{51} as sodium chromate in 2 ml of solution. The tagged aliquot was frozen by the standard technic described above under experimental procedure. Fifty ml of plasma were removed from the untagged aliquot and used to cover the bottom of the reconstitution vessel. Because of the unaccustomed attempt to maintain a semblance of sterile technic, portions of the thawing procedure were clumsily handled and 6% hemolysis resulted. An aliquot of this blood containing an estimated 10 ml of cells was reinfused into the original donor by intravenous drip. Other than a brief psychosomatic response, no adverse clinical reaction was observed. Samples withdrawn 5, 10 and 15 minutes following infusion showed no change in radioactivity. A normal red cell volume calculated from these values indicated no immediate loss of cells after injection. At 6 hours a fall of 6% was observed. Subsequent samples taken daily for a week and at weekly intervals thereafter showed a slightly accelerated biological decay during the first 2 weeks with normal survival thereafter. (Table I). A second transfusion, this time homologous, is also charted in Table I. Mechanical failure again dogged the thawing apparatus and was presumably responsible for much of the 7% spontaneous hemolysis. Subsequent biological decay was again accelerated. Between the second and third week there was virtually a complete loss of blood radioactivity. A battery of tests failed to demonstrate any antigen in the donor's erythrocytes which might have been responsible. Preliminary experiments in rabbits indicate

that there may be an alteration in the attachment of chromium to the cell when tagging is done prior to freezing and thawing.

An *in vitro* examination of the blood platelets showed them to be substantially reduced in number as anticipated inasmuch as special precautions for platelet handling were not observed. Those remaining in whole blood after freezing and thawing appeared morphologically normal but were present in insufficient number for any test of function.

The following determinations were made on the clotting factors of the supernatant plasma: prothrombin time, recalcification time, accelerator globulin, antihemophilic factor, plasma thromboplastic component and serum prothrombin conversion accelerator. All were unaltered within the limits of experimental error compared to determinations on the same blood prior to freezing and thawing.

Storage. Only the most preliminary storage experiments have been undertaken. X-ray diffraction studies show complete crystallization in the frozen blood on the basis of which it is assumed that, while storage below about 130°C would probably be virtually indefinite, higher and more practical temperatures should still permit good survival for months or years, limited primarily by the rate of denaturation resulting from the dehydration of freezing. Exposures to temperatures as high as −20°C for a few minutes were found to be without ill effect. Preliminary results from longer storage at −60°C are difficult to interpret because of an accompanying acidification due to exposure to CO_2 from the dry ice coolant. Storage loss at −60°C was initially rather rapid, reaching 15% after 20 days (pH 6.5) but slowing subsequently to less than 2% per week. There is every expectation that the relationship between storage decay and temperature will be in accordance with the Arrhenius expression, showing a logarithmic decrease in decay rate with reduction in temperature.

Conclusions. Aside from premature speculation on the application of rapid freezing to blood preservation and storage, it can be unequivocally stated that, on the basis of *in vivo* survival, erythrocytes may be taken to liquid nitrogen temperature and back without destruction. The rather unusual fragility of the blood cell and the close relationship between its fragility and its state of health supports the impression that erythrocytes are particularly difficult to freeze. This in turn lends encouragement to the hope that other biological entities which can meet the rather stringent size requirements can be similarly frozen making available not only the benefits of long term storage, but providing also an intriguing new research tool through the study of transient phenomena interrupted and immobilized by the solid state.

Summary. 1. In accordance with previously derived criteria for non-traumatic rapid freezing, very small (.45-.9 mm) droplets of citrated whole blood with added dextrose are sprayed onto liquid nitrogen. The frozen blood is reconstituted in warm saline. 2. Two human transfusions of frozen and thawed whole blood containing erythrocytes tagged with Cr^{51} demonstrate the ability of red cells to survive *in vivo* after freezing in liquid nitrogen at −195.8°C.

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