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## PLATELET PRESERVATION. II. PRESERVATION OF CANINE PLATELET CONCENTRATES BY FREEZING IN SOLU- TIONS OF GLYCEROL PLASMA \*

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The successful long-term preservation of bull semen by freezing in glycerol solutions provided the impetus for application of this technique to other cells and tissues (1). Among the hematopoietic elements, the red blood cells (2-7) and the bone marrow (8) have been preserved by this method. In the case of bone marrow, evidence that a high percentage of viable cells emerges from glycerol freezing is lacking, since there is no adequate quantitation of a transfused bone marrow dose-response curve. A few viable primordial elements of the marrow (reticulum cells, hemocytoblasts) may be adequate to provide a "take." By analogy only a small percentage of viable bull sperm may be required to cause conception. Only in the case of red blood cells is there evidence in man and laboratory animals that a high percentage of cells (90 per cent) survives glycerol freezing and is capable of living a normal life span after transfusion into a compatible recipient (7).

The red blood cell in several respects lends itself readily to *in vitro* manipulation. It survives the trauma of contact with foreign surfaces, anticoagulation (9), centrifugation, acidification (10), cooling (4° C), supercooling (-3° C) (11), and warming (4° to 37° C). Despite bizarre morphology and ample evidence of chemical alteration with storage (12), the red blood cell survives the lesion incurred in leaving the body by its remarkable capacity to be reconstituted on renewal of contact with the blood stream (13, 14).

Platelet preservation by glycerol freezing (15-20), however, has lagged behind that of the red blood cell for several reasons. Platelets are not handled *in vitro* with the same facility as red blood cells. The very nature and function of the platelet renders it susceptible to irreversible damage

from the moment it leaves its intravascular milieu (21-23). This irreversible damage may shorten the platelet life span without altering its morphological appearance or clot-retraction function (18, 24-26). Any worthwhile preservation procedure, therefore, must retain in the preserved platelets the capacity to survive normally *in vivo* (16-19, 25, 26) as well as the capacity to perform clot-retraction and thromboplastic functions *in vitro* (27, 28).

The canine was selected as the animal model for development of platelet preservation techniques. This animal offers several advantages over smaller animals. Volumes of blood nearly comparable (350 to 400 ml) with those obtained in standard phlebotomies in humans (450 to 500 ml) are obtained with ease, thus permitting experience in handling platelets comparable in volume with that anticipated in man. The dog is relatively long-lived compared with smaller laboratory animals, permitting observations in the same animal over many years. This has permitted a linear experience with various methods for platelet preservation using the same animal over and over again to serve as its own control (16, 18, 25, 26). Some of the dogs have had 40 or more platelet transfusions over a period of 5 years.

The Cr<sup>51</sup> technique for labeling human platelets (29, 30) was adapted to dogs (25, 26) and in this and previous reports from this laboratory has been used as the major criterion for platelet viability after glycerol freezing (16, 18). All of the glycerol experiments were performed with platelet concentrates rather than whole blood or platelet-rich plasma. The goal in the canine experimentation has been to translate the data into clinical application. Because of the volume relationships in platelet replacement therapy with whole blood and its components (31), it has been decided to strive for preservation of platelet concentrates.

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The ease of handling platelets in a plastic bag system has permitted the good results in obtaining viable platelets for therapeutic or radioisotope-labeled transfusions (29-33). This plastic bag system has been used in all of the studies reported here and has provided a closed, sterile system for addition of glycerol and freezing and thawing, without necessity for transferring the platelets during the procedure. Polyvinyl chloride plastic has been demonstrated to be capable of tolerating extremes of temperature without altering its hemorepellency or surface continuity.

#### MATERIALS AND METHODS

*Cr<sup>51</sup>-platelet life span.* The techniques for labeling canine platelet concentrates and measuring their life span have been described in Part 1 of this report (25). Previous experience with canine platelets has been with concentrates prepared from blood anticoagulated either with acid citrate dextrose (ACD-NIH formula) or ethylenediamine tetraacetic anhydride disodium (EDTA) (25). Since human platelet concentrates almost invariably clump when prepared from ACD blood, it was decided to gain experience in the canine with an anticoagulant that would be applicable to man (EDTA).

*Recording results.* In this report canine Cr<sup>51</sup>-platelet life span has been plotted on arithmetic graph paper with time on the abscissa. On the ordinate the per cent of the highest recoverable platelet-bound radioactivity from daily 12-ml samples of whole blood has been graphed. This method has permitted comparison between studies involving the manipulation of several variables. Each variation in technique was repeated at least six times in separate animals. The results recorded in this report summarize the experience with some 400 Cr<sup>51</sup>-platelet transfusions (normal and glycerol-treated) in the same colony of dogs. Because the same labeling of Cr<sup>51</sup> was used in all of the studies reported, the per cent method of graphing has had validity, since comparable recovery of platelet-bound radioactivity has been obtained through many variations in preservation procedures. When the counts per minute recovery in all platelet samples was low this type of curve was recognized and has been referred to as a diminutive curve in a previous report from this laboratory (30).

The platelet viability index of Baldini, Costea and Dameshek (19) cannot be used effectively to graph the results of glycerol-freezing experiments. One of the difficulties inherent in glycerol freezing is the hyperosmolarity of glycerol-treated cells. This hyperosmolarity not only can influence the *in vivo* survival of transfused cells (red blood cell or platelets, for example) but also can lead to osmotic rupture with *in vitro* exposure to isotonic saline. The denominator of the platelet viability index depends upon the radioactivity of a twice saline-washed aliquot of platelets from the donor sample. Glycerol-treated hyperos-

motric platelets cannot survive exposure to large volumes of isotonic saline without some attrition by osmotic lysis. The loss of radioactivity caused by osmotic lysis may lower the value of the denominator in the viability index. If less administered radioactivity has to be accounted for, the apparent per cent yield may be higher.

*Glycerol addition.* Glycerol (reagent grade, Merck) has been added directly to Cr<sup>51</sup>-labeled platelet concentrates. The glycerol has withstood treatment with pyrogen-absorbing charcoals<sup>1</sup> and autoclaving without change in its appearance or effectiveness in preservation experiments. This glycerol reagent was added to platelet concentrates both directly (99.6 per cent glycerol) and in a variety of solvents. The electrolyte solvents used were 0.85 per cent saline, Ringer's solution, Sorensen's phosphate buffer (pH 7.4), and 0.167 M sodium lactate. The plasma substitutes and derivatives used as solvents were gelatin, modified fluid gelatin,<sup>2</sup> stored human plasma, plasmanate,<sup>3</sup> human salt-poor albumin, human  $\gamma$ -globulin, 6 per cent dextran in saline (of low<sup>4</sup> and high<sup>5</sup> mean mol wt), and autologous platelet-poor plasma. Glycerol was prepared daily as a 30 per cent solution by volume in one of the solvents listed above. The glycerol was subjected to autoclaving only when dissolved in one of the electrolyte solutions.

The volume of the platelet concentrate was determined by weighing the plastic bag containing the concentrate and subtracting the known weight of an empty bag. The glycerol then was added by volume as  $\frac{1}{6}$ ,  $\frac{1}{5}$ ,  $\frac{1}{4}$ ,  $\frac{1}{3}$ ,  $\frac{1}{2}$ ,  $\frac{2}{3}$  of the volume in milliliters (weight in grams) of the concentrate to achieve final concentrations of 4.5, 5, 6, 7.5, 10, and 12.5 per cent glycerol (vol/vol). The entire amount of glycerol was added by direct, rapid injection into the platelet concentrate. Slower, dropwise addition of glycerol (over a period of 5 minutes) offered no apparent advantage. The glycerol-treated platelet concentrates were incubated at room temperature for periods ranging from 1 to 20 minutes. The majority of experiments were performed with a 20-minute glycerol incubation time.

A separate group of experiments was done in which 7.5 per cent glycerol (vol/vol) and dextrose, 12 ml of a 35 per cent solution in platelet-poor plasma, were added to the platelet concentrate simultaneously. After the usual 20-minute incubation period the mixture was frozen and thawed.

*Method of freezing.* The freezing was performed in two ways.

1) The glycerol-treated platelet concentrate was kept in the original plastic bag into which the platelet-rich plasma had been transferred from the whole blood. A

<sup>1</sup> Decolorizing charcoal, Mallinckrodt.

<sup>2</sup> Modified fluid gelatine 3% in 0.7% NaCl, lot MFG-10, Knox Gelatine Co.

<sup>3</sup> Plasmanate, plasma protein fraction, 5% (human), Cutter Labs.

<sup>4</sup> Mean mol wt 70,000; Abbott Labs.

<sup>5</sup> Mean mol wt 190,000; (Intradex) Glaxo Labs., Greenford, England.

thermistor probe,<sup>6</sup> range  $-68^{\circ}$  to  $+10^{\circ}$  C, connected with a temperature recorder,<sup>6</sup> was inserted into the center of the platelet concentrate through the plastic inlet tubing of the bag. The plastic bag then was placed upright on the bottom shelf of an upright deep freeze. The door of the deep freeze was closed on the thin wire connecting recorder and thermistor. A temperature curve was recorded by 1-minute readings from the recorder. When the temperature dropped to  $-5^{\circ}$  C the bag was transferred rapidly (within 10 seconds) into a large, heavily insulated box packed with dry ice at an average air temperature of  $-75^{\circ}$  to  $-79^{\circ}$  C. The temperature of the platelet concentrate was recorded until it reached  $-70^{\circ}$  C.

2) The glycerol-treated platelet concentrate was kept in the original plastic bag into which the platelet-rich plasma had been transferred from the whole blood. The bag then was suspended in a methyl alcohol bath of 0.5 gallon capacity, which in turn was connected with a dry ice-methyl alcohol mixture in a 30-gallon reservoir.<sup>7</sup> An electronically cam-operated recorder<sup>8</sup> controlled a pump which delivered methyl alcohol at  $-79^{\circ}$  C to the freezing bath. Two cams were used, one with a  $1^{\circ}$  C per minute temperature decrement (in the bath) from  $+20^{\circ}$  to  $-30^{\circ}$  C followed by a  $5^{\circ}$  decrement from  $-30^{\circ}$  to  $-70^{\circ}$  C; the other with a  $1^{\circ}$  per minute decrement from  $+20^{\circ}$  to  $-70^{\circ}$  C. The cams were controlled by a steel thermoprobe (accuracy  $\pm 0.25^{\circ}$  C) set in the jet intake of the pump so that overshooting was minimized. The chief differences between this freezing apparatus and those used in other laboratories are the large reservoir of refrigerant and the critical location of the thermoprobe control. Temperature curves were recorded by a direct-writing pen connected with the cam and responsive to the thermoprobe. These curves were filed separately with each experiment as a permanent record.

The thermistor probe also was inserted into the plastic bag in the experiments with the electronic control system. From these experiments comparison between the temperature decrements in the refrigerant bath and the platelet concentrate was obtained (Figure 1).

The majority of the experiments reported here were performed with the electronic control system in order to provide for reproducibility. Experiments also were done in which the rate of freezing of glycerol-treated platelet concentrates was  $5^{\circ}$  and  $10^{\circ}$  C per minute for the entire freezing process. On two occasions glycerol-treated platelet concentrates were frozen by plunging into a dry ice-methyl alcohol bath, reaching equilibrium at  $-70^{\circ}$  C in 1 minute.

Six experiments were performed with nonglycerol-treated platelet concentrates which were frozen at  $1^{\circ}$  C per minute from  $+20^{\circ}$  to  $-79^{\circ}$  C.

*Method of storage.* The plastic bag was removed from the freezing bath and placed in a rigid cardboard container that was placed at the bottom of a well insulated

dry ice storage container in which a constant level of dry ice was maintained, providing an average air temperature of  $-75^{\circ}$  C.

*Method of thawing.* The methods of thawing used were quite simple.

1) The plastic bag containing the frozen glycerol-treated platelet concentrate was removed from the freezing bath or dry ice storage container and placed under running cold tap water until thawing occurred in 5 to 7 minutes. 2) The plastic bag was plunged into a  $37^{\circ}$  C water bath until thawing occurred in 3 to 4 minutes. 3) More rapid rates of thawing (brief exposure to water heated above  $37^{\circ}$  C, for example) were not attempted. 4) The rate of thawing was recorded in several experiments by measurement of temperature changes within the thawing platelet concentrate by an indwelling thermistor probe (Figure 2).

*Method of removing glycerol from or effecting osmotic buffering for thawed glycerol-treated platelet concentrates.* Dextrose and sorbitol were dissolved in the same series of solvents as those used for glycerol. The dextrose and sorbitol were prepared as a 17.5, 25 or 35 per cent solution in the various solvents. Where electrolyte solvents were used, the dextrose was dissolved in a volume of sterile solvent and autoclaved. All other dextrose solutions were prepared without autoclaving on the day of use.

Thirty-five per cent dextrose dissolved in autologous platelet-poor plasma was used most frequently; 3 ml of this solution was added directly to the thawed, glycerol-treated concentrate, mixed, and equilibrated for 2 minutes. This was repeated three more times until 12 ml had been added to the platelet concentrate. This resulted in a 10 to 12 per cent dextrose concentration in the transfusion mixture.

Seventeen and one-half per cent dextrose in platelet-poor plasma was added in a slightly different manner; i.e., 4 ml of solution every 2 minutes until 24 ml had been added to the platelet concentrate. This permitted the addition of larger volumes of glycerol-free plasma to achieve an 8 to 10 per cent concentration of dextrose in the transfusion mixture.

Removal of glycerol was attempted by centrifugation of the thawed glycerol-treated platelets at 1,000 G for 30 minutes at  $2^{\circ}$  C. The glycerol containing supernatant platelet-poor plasma was decanted. The glycerol-treated platelet concentrate was resuspended in one-half of the concentration of glycerol plasma originally employed before freezing, or in a 12-ml volume of 17.5 or 35 per cent dextrose in the manner described above. In some instances a second centrifugation was performed to separate more glycerol-containing plasma from the platelet concentrate. This second centrifugation was followed by attempts to resuspend the platelet concentrate in one-fourth of the concentration of the glycerol plasma originally employed or in 12 ml of 17.5 or 35 per cent dextrose in platelet-poor plasma.

*Method of transfusion and sampling.* The platelet transfusions were infused into the jugular vein through a short plastic adapter and a no. 18 needle. The average

<sup>6</sup> Fenwal Inc., Framingham, Mass.

<sup>7</sup> Harris Refrigeration Co., Cambridge, Mass.

<sup>8</sup> Bristol Co., Waterbury, Conn.

time for administering a platelet transfusion of 30- to 50-ml volume was 30 to 60 seconds. To estimate the life span of the labeled platelets, 12-ml blood samples were obtained from the jugular vein at 0.5 and 2 hours and daily thereafter until no radioactivity was detectable in the recipient's platelets. Platelets were harvested from these blood samples by differential sedimentation, using 6 per cent dextran, as reported previously from this laboratory (30).

### RESULTS

*Normal Cr<sup>51</sup>-platelet life span.* The characteristics of the life-span curve of Cr<sup>51</sup>-labeled canine platelet concentrates have been discussed in Part I of this report (25).

*Freezing of glycerol-treated platelet concentrates.* The optimum rates of freezing were determined by modifying the temperature decre-

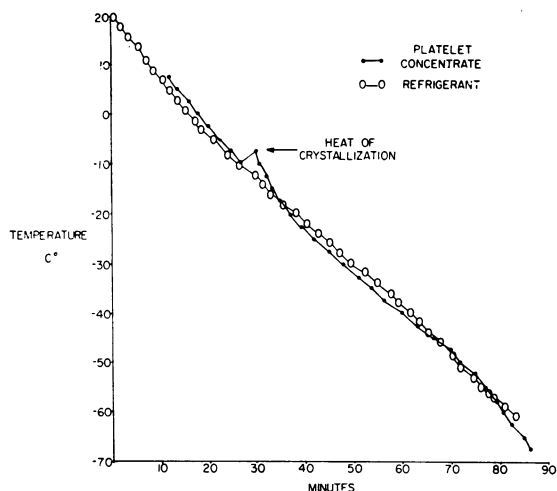


FIG. 1. COMPARISON BETWEEN THE TEMPERATURE DECREMENTS IN THE REFRIGERANT BATH AND PLATELET CONCENTRATE. The ordinate shows the temperature decrements (+20° through -70° C). The abscissa shows the time in minutes. The temperature decrement of the platelet concentrate (closed circles) followed the temperature decrement of the refrigerant bath (open circles) until -9° C, at which point a sudden rise in the temperature of the platelet concentrate was noted (heat of crystallization). This increase in temperature was sustained for approximately 3 minutes during which time ice crystal formation presumably was completed. The temperature within the platelet concentrate then fell at a faster rate in order to reach equilibrium with the refrigerant bath at -16° C. From -16° C the temperature decrements in the platelet concentrate and the refrigerant bath again were parallel. The curves shown here were made from one experiment but are representative of those obtained consistently with the electronically controlled freezing method.

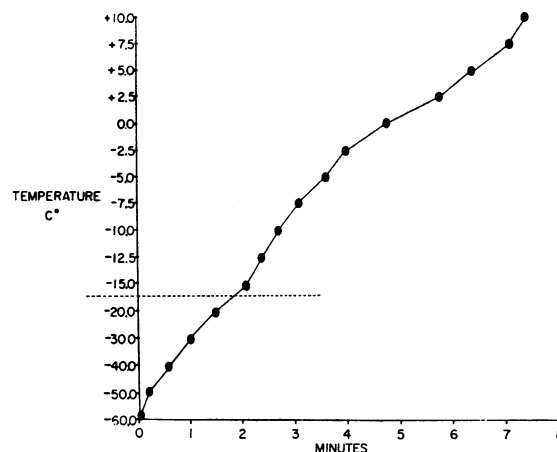


FIG. 2. RATE OF THAWING OF PLATELET CONCENTRATE INDUCED BY RUNNING COLD TAP WATER. The ordinate shows the temperature increments (-60° through +10° C). The abscissa shows the time in minutes. The temperature recordings were made from within the thawing platelet concentrate by a thermistor probe which had been inserted prior to freezing.

ments in the refrigerant surrounding the container for the platelet concentrate. These were related to the temperature changes within the platelet concentrate (Figure 1). The temperature decrements in the refrigerant paralleled the changes in the platelet concentrate from the starting point (+20° C) to the point of ice-crystal formation in the platelet concentrate. Glycerol addition permitted supercooling to -3° to -9° C before ice crystallization occurred. At the time of crystallization there was an immediate 3° to 5° C rise in temperature of the platelet concentrate, which was maintained for approximately 2 minutes. During this period the temperature of the surrounding refrigerant continued to decline to -10° to -11° C, compared with -5° C within the solidifying platelet concentrate. When solidification was completed the temperature within the platelet concentrate declined at a rapid rate (3° to 4° C per minute) until it reached -16°, at which time it again paralleled changes in the surrounding refrigerant (Figure 1). This type of freezing curve was reproducible and in all subsequent figures it may be assumed that the temperature curve generally followed that depicted in Figure 2.

*Glycerol addition.* Controlled slow freezing (1° C per minute from +20° to -70° C) of platelet concentrates which were not treated with glycerol resulted in complete destruction of the

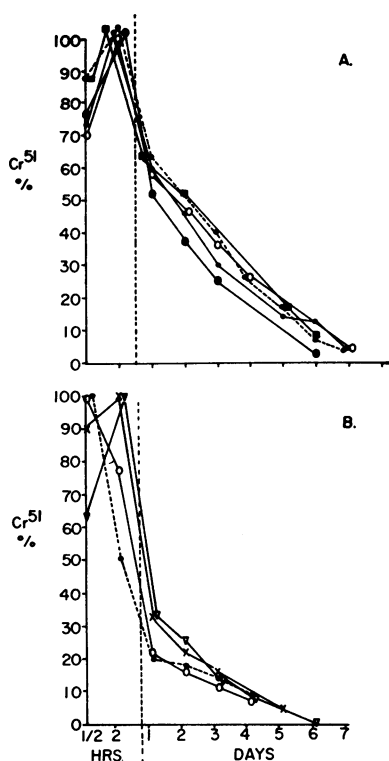


FIG. 3. FOR BOTH SERIES OF CURVES THE  $\text{Cr}^{51}$ -PLATELET LIFE SPAN WAS PLOTTED WITH PER CENT OF HIGHEST RECOVERABLE PLATELET-BOUND RADIOACTIVITY ON THE ORDINATE AND TIME ON THE ABSCISSA. **A.** The effect of glycerol addition alone on the  $\text{Cr}^{51}$ -platelet life span was determined. Comparison of this series of curves with those of Figure 1 in Part I of this report demonstrates a deleterious effect on platelet viability by glycerol addition without freezing. The normal  $\text{Cr}^{51}$ -platelet life-span curves showed approximately 80 per cent of recoverable platelet-bound radioactivity on the first day after transfusion. The  $\text{Cr}^{51}$ -labeled glycerol-treated platelet concentrates, however, showed an average of 60 per cent of recoverable platelet-bound radioactivity on the first day after transfusion. **B.** The effect of glycerol addition plus freezing and thawing on the  $\text{Cr}^{51}$ -platelet life span was determined. Comparison with the upper series of curves shows a marked loss of platelet viability. An average of 30 per cent of recoverable platelet-bound radioactivity was found on the first day after transfusion.

administered platelets, as determined by  $\text{Cr}^{51}$  life span. This experiment was repeated with the exception that the platelet concentrate was incubated with glycerol (final concentration 7.5 per cent by volume) without freezing. Transfusion of the glycerol-treated platelet concentrate resulted in a diphasic  $\text{Cr}^{51}$  life-span curve. After an initial good recovery of platelet-bound radioactivity

in the 0.5- and 2-hour post-transfusion samples there was a sharp decline in recoverable platelet radioactivity in the 24-hour sample (averaging 60 per cent of the peak level of radioactivity) followed by a normal slope of decline and a 7-day life span (Figure 3A).

Glycerol-treated platelet concentrates which were frozen and thawed showed a marked loss of platelet viability, with the 24-hour post-transfusion sample averaging only 30 per cent of the peak level of radioactivity compared with 60 per cent on the unfrozen studies. When the freezing process was more rapid than  $2^\circ \text{C}$  per minute, there was virtually complete destruction of  $\text{Cr}^{51}$  glycerol-treated platelet concentrates.

*Thawing.* The rate of thawing was determined by temperature recordings from within the glycerol-treated platelet concentrate (contained in a plastic bag) immersed in a  $37^\circ \text{C}$  water bath (Figure 2). Slower rates of thawing (cold tap water) offered no apparent advantage.

*Osmotic buffering of glycerol (addition of hypertonic dextrose and sorbitol).* Addition of 35 per cent dextrose solutions to glycerol-treated platelet concentrates that were not frozen permitted a normal  $\text{Cr}^{51}$ -platelet life-span pattern (Figure 4A). The glycerol-treated platelet concentrates that were frozen and thawed were incompletely protected by dextrose (Figure 4B). The life-span studies in this group showed an average of 65 per cent platelet-bound radioactivity in the 24-hour blood sample, compared with an average of 80 per cent activity in the control (unfrozen) studies (Figure 4A).

In order to increase the volume of glycerol-free plasma available for dilution of intracellular glycerol, the dextrose was added in half the concentration (17.5 per cent in platelet-poor plasma) and twice the volume (24 ml). This resulted in  $\text{Cr}^{51}$ -platelet life-span patterns which were similar to those with the 35 per cent dextrose. Thirty-five per cent and 17.5 per cent sorbitol in platelet-poor plasma were found to substitute effectively for the dextrose solutions in the studies mentioned.

*Removal of glycerol.* In almost all of the experiments in which glycerol removal was tried, the platelet concentrates could not be resuspended in the hypertonic dextrose or in glycerol plasma solutions. Large clumps of platelets, which were not seen with normal or other glycerol studies, were

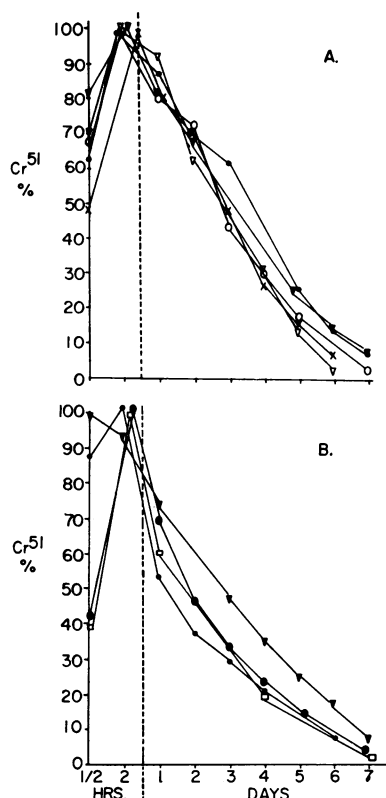


FIG. 4. FOR BOTH SERIES OF CURVES THE  $\text{Cr}^{51}$ -PLATELET LIFE SPAN WAS PLOTTED WITH PER CENT OF HIGHEST RECOVERABLE PLATELET-BOUND RADIOACTIVITY ON THE ORDINATE AND TIME ON THE ABSCISSA. **A.** The effect on  $\text{Cr}^{51}$ -platelet life span of adding hypertonic dextrose to glycerol-treated platelet concentrates was determined. The series of curves obtained had the characteristics of a normal  $\text{Cr}^{51}$ -platelet life span (Figure 1 in Part I of this report). **B.** The effect of adding hypertonic dextrose to glycerol-treated platelet concentrates which had been frozen and thawed was determined. Comparison with the series of curves in Figure 3B demonstrates the protective effect of hypertonic dextrose. The series of curves in Figure 4B is comparable with those obtained in Figure 3A where glycerol was added but freezing was not done. The addition of hypertonic dextrose to glycerol-treated platelet concentrates which had been frozen and thawed did not, however, completely preserve platelet viability. Comparison with the upper series of curves shows a moderate loss of platelet viability. An average of 65 per cent of recoverable platelet-bound radioactivity was found on the first day after transfusion.

present and tended to enlarge as further efforts were made to suspend them. When twice the volume of 17.5 per cent dextrose was added the packed glycerol-treated platelets resuspended more easily, but in most experiments gross clumping still resulted.

*Short-term storage of glycerol-treated platelet concentrates.*  $\text{Cr}^{51}$ -labeled, glycerol-treated platelet concentrates were stored at  $-75^{\circ}\text{C}$  for periods of 3 to 42 days. At the end of the storage period the platelet concentrates were thawed, and dextrose dissolved in autologous platelet-poor plasma was added as previously described. When these stored platelet concentrates were administered to the original donor animals, the  $\text{Cr}^{51}$ -platelet life spans were identical with those obtained with concentrates that were thawed immediately upon completion of the freezing process.

#### DISCUSSION

The success reported here with preservation of canine platelets by controlled slow freezing in glycerol plasma represents a fortuitous combination of procedures, many of which may not apply to glycerol preservation of human platelets (17, 18). The procedures used were pragmatically applied, based on the collective experience of other investigators working with glycerol preservation techniques applied to bone marrow (8) and red blood cells (2-7, 34). No attempt has been made in this report to prove the exact mechanism of action of glycerol in protecting canine platelets against the harmful effects of freezing.

*Glycerol addition.* Glycerol penetrates the cell interior with a facility that is disproportionate to its molecular size (35). This may be due to its three hydroxyl groups that may have an affinity for hydroxyl groups in the cell membrane or cell interior; or it may be related to the lipid-water solubility relationships of the glycerol molecule, favoring easy penetration of the lipid-rich cell membrane. Whatever the mechanism, the penetrability of glycerol probably is responsible for its usefulness in low temperature preservation work. Once inside the cell the hydrophilic capacities of glycerol are put to use to protect the cell against the harmful effects of freezing. Although no direct attempts have been made to prove the mode of cell penetration or mode of action of glycerol in protecting platelets against freezing, certain observations have been made empirically that may elucidate some of the basic mechanisms.

The concentrations of glycerol used in platelet (15-20), red blood cell (2-7), and bone marrow (8) techniques have varied from 5 to 50 per cent.

Concentrations of glycerol of 12 per cent or more have not permitted *in vivo* canine platelet survival before or after freezing, with or without osmotic buffering by hypertonic dextrose. Concentrations of glycerol of less than 4.5 per cent had no effect on canine Cr<sup>51</sup>-platelet life span without freezing but conferred no protection during freezing. The optimum concentration of glycerol, vol/vol, was in the 7.5 to 10 per cent range.

The optimum solvent for preparing glycerol reagents proved to be autologous platelet-poor plasma. In addition to providing a physiological buffer at pH 7.4, plasma probably also provided a protein coating for platelets (36, 37), since no electrolyte solution conferred the same protection.

The speed of glycerol addition to the platelet concentrate made no apparent difference in the Cr<sup>51</sup> life span. Dropwise addition of glycerol did not enhance the survival of glycerol-treated frozen platelets over that obtained with injection of the entire amount of glycerol as a single bolus. The time necessary for equilibration with glycerol was not evaluated thoroughly.

*Freezing.* The methods of freezing used were on the one hand more exact, and on the other more crude, than those used by other investigators in this field (1-8, 15, 16, 19, 20). The electronically controlled freezing apparatus gave remarkably reproducible freezing curves, permitting accurate standardization of this variable. The deep freeze-dry ice box, air-freezing method was used for 1 year prior to the use of the electronically controlled method. The results with the casual air-freezing method have not been bettered.

All slow-freezing methods with prior glycerol addition must take into account the distortion of the freezing curve produced by supercooling and the heat of crystallization (38), the former produced by glycerol, the latter due to heat production during the ice crystal formation. These distortions were observed when temperature recordings were made from within the freezing platelet mixture (38). The introduction of the thermoprobe into the plastic bag caused bacterial contamination of the platelet concentrate, and precludes similar observations in man. Comparison of bath temperature and platelet concentrate temperature during slow freezing emphasized that in all future work with low-temperature preservation, translation of

bath temperature curves into tissue or intracellular temperature curves is to be discouraged (Figure 2). The bath temperature is merely an arbitrary guide and not the basis for postulating physiological limits of stress in low-temperature preservation work.

*Osmotic buffering of intracellular glycerol.* Osmotic buffering of thawed glycerol-treated platelet concentrates was necessary in order to achieve maximal platelet yields after transfusion. The two agents used were dextrose and sorbitol. The former requires active transport to penetrate the cell interior (unlike glycerol), and for this reason is able to effect an osmotic gradient favoring exosmosis of water from the cell (39-42). Sorbitol, on the other hand, does not penetrate the cell interior at all (43) and gram for gram exerts a stronger extracellular osmotic gradient. Both of these sugars in hypertonic solutions, varying from 8 to 12 per cent in the final transfusion mixture, have been effective in shrinking the glycerol-swollen platelets to normal or even less than normal size. Presumably the shrinkage has been due to an exosmosis of free water and glycerol from inside the cell. Because the platelets have been osmotically more competent after addition of these sugars it has been assumed that the flux of water is accompanied by a passive flux of the freely penetrating glycerol molecule into the extracellular fluid. Although complete glycerol removal has not been achieved by this technique it represents a far more gentle and potentially useful technique for platelet work than the glycerol removal procedures reported by Sloviter (34) and Tullis and co-workers (7). Apparently the glycerol, which is held extracellularly by an osmotic gradient at the time of transfusion, is diluted by the circulating plasma and cannot repenetrate the platelet. The glycerol itself is metabolized rapidly (44). Each platelet transfusion in these experiments contained 2 to 3 g (30 to 40 ml of 7.5 per cent glycerol solution) of glycerol. Sorbitol is metabolized as rapidly as dextrose and is safer to administer to man than is sucrose which, although osmotically effective, is potentially harmful to the kidneys (45). Other disaccharides such as lactose (20) should be used with caution, since their renal or other toxicity in man is not known.

Addition of sorbitol or dextrose and glycerol to-



gether prior to freezing, however, greatly decreases the yield of viable platelets. Platelets so treated were smaller and presumably more dehydrated at the time of freezing. The dehydration itself may have augmented the lesion of freezing. This lesion has been attributed to enzyme death produced by hypertonicity of the interstitial fluid remaining between ice crystals, the latter having been formed from solute free water. This observation also reinforces the observations of Lovelock who suggested that lowering the intracellular osmolarity prior to freezing would ameliorate the harmful effects of hypertonicity during the freezing process (46). It has been assumed that sorbitol or dextrose addition prior to freezing increased intracellular osmolarity by causing exosmosis of intracellular water. In addition, this observation may explain the harmful effects of glycerol concentrations exceeding 12 per cent. If that portion of glycerol which remained extracellularly caused water to leave the cell interior to be bound to extracellular glycerol, then the latter would have had a dehydrating effect on the intracellular fluid.

#### SUMMARY

Canine platelet concentrates have been preserved by an adaptation of the glycerol freezing technique. The optimum concentration of glycerol was 7.5 to 10 per cent, vol/vol. Glycerol-treated platelet concentrates which were frozen and thawed showed a marked loss of platelet viability with the 24-hour post-transfusion sample averaging only 30 per cent of the peak level of radioactivity, compared with 60 per cent in the unfrozen studies.

Gentle, partial removal of glycerol was achieved by addition of hypertonic dextrose or sorbitol to the thawed glycerol-treated platelets. Presumably, these sugars in hypertonic solution achieved a passive flux of glycerol with the exosmosis of water to achieve osmotic equilibrium. Addition of 35 per cent dextrose or sorbitol solutions to glycerol-treated platelet concentrates that were not frozen permitted a normal  $\text{Cr}^{51}$ -platelet life-span pattern. The glycerol-treated platelet concentrates that were frozen and thawed were incompletely protected by hypertonic dextrose or sorbitol. The life-span studies in this group showed an average of 65 per cent platelet-bound radioactivity in the 24-hour

blood sample, compared with an average of 80 per cent activity in the control (unfrozen) studies.

Glycerol addition permits supercooling of solutions. The supercooling effect and the heat of crystallization caused a considerable distortion of the "ideal"  $1^\circ \text{C}$  per minute temperature decrement curve. The temperature of the refrigerant bath was particularly misleading during the early portion of the critical zone of injury ( $-9^\circ$  to  $16^\circ \text{C}$ ).

The thawing procedures were simple, involving immersion of the plastic bag containing the frozen platelets in water at  $22^\circ$  or  $37^\circ \text{C}$ .

The maintenance of a plasmatic milieu was found to be essential for optimum success of the method. The glycerol, dextrose, and sorbitol, therefore, were dissolved in autologous platelet-plasma.

The method of assessing *in vivo* viability of frozen and thawed, glycerol-treated platelet concentrates was the life span of platelet concentrates labeled with  $\text{Cr}^{51}$  before glycerol addition. Sufficient experience with the  $\text{Cr}^{51}$ -labeling method has been achieved in this laboratory to permit evaluation of *in vivo* viability by this parameter alone. The number of experiments upon which the report is based further enhances the statistical validity of this conclusion.

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