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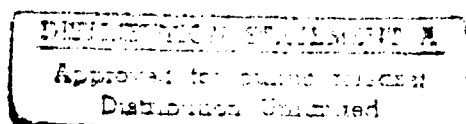


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July 12, 1991



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RESEARCH PROGRESS REPORT  
SUBMITTED TO THE  
U.S. NAVAL MEDICAL RESEARCH AND DEVELOPMENT COMMAND

FREEZE-DRIED HUMAN RED BLOOD CELLS

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CRYOPHARM CORPORATION  
July 12, 1991

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### Section 2

#### CURRENT CLINICAL PROTOCOL AND METHODS

### Section 3

DRAFT MANUSCRIPT:  
"Metabolic Activities of Freeze-Dried Human Erythrocytes"

## SUMMARY

This research report focuses on Cryopharm's second clinical study, and summarizes our progress on lyophilized human red blood cells since the last progress report submitted on March 15, 1991.

In the second study, low doses (10-15 ml) of packed  $^{51}\text{Cr}$ -labeled autologous red cells were infused into four normal volunteers following lyophilization and rehydration of the cells. The design of the clinical study protocol (Section 2) followed accepted procedures for red cell sequestration studies to evaluate splenic function or to diagnose hemolytic anemias (Ref. 1-3). In all four volunteers no changes in vital signs occurred after infusion of the autologous lyophilized red cells, and no associated adverse side effects were observed during the study, through the final follow-up examination conducted one week post-infusion.

In all four volunteers, peripheral blood samples were collected for up to five days post-infusion to measure the level of circulating  $^{51}\text{Cr}$ . Urine collections during the first 24 hours post-infusion were also counted to measure the level of chromium clearance via the kidneys. Each volunteer was subjected to gamma camera imaging (including the lungs, which do not accumulate the isotopic label) and external probe counts over the heart, spleen, and liver at 4 hours and 24 hours post-infusion, to ascertain the organ distribution of the injected isotopic label over time.

The data from this study indicate that  $^{51}\text{Cr}$ -labeled lyophilized reconstituted red cells are rapidly cleared from peripheral circulation, in spite of the improved osmotic stability and overall deformability of the cells relative to our first clinical study in October 1990. However, the mechanism of clearance as elucidated by organ distribution appears to predominantly involve splenic sequestration of intact cells, instead of the rapid intravascular hemolysis observed in October 1990. In brief, it appears that the lyophilized red cells used in this study are capable for the most part of remaining intact following infusion and initial circulation through the heart, lungs, and major vasculature, but cannot yet survive passage through the splenic fenestrae (the fenestrae are interendothelial slits through which red cells must traverse in order to flow from the terminal arterioles in the spleen to the venous sinuses; Ref. 4).

The splenic fenestrae are thought to clear red cells by two principle mechanisms: 1) antibody-sensitized red cells are engulfed by phagocytic cells in the spleen, and 2) cells with reduced deformability or abnormal morphology (spherocytes) are trapped in the fenestrae and subsequently ingested by phagocytic cells (Ref. 4). In each clinical volunteer we did not detect significant binding of autoantibodies to autologous lyophilized red cells, using standard clinical crossmatching (this procedure uses IgG directed against human IgG or complement to detect binding of sensitizing

antibodies to the red cell membranes). We therefore believe that cell deformability or other cell membrane properties may still play a significant role in the observed clearance of the cells. As we will discuss in the Future Plans section of this report, the possibility of mild sensitization by autoantibodies can also be addressed.

Our basic research effort during the next reporting period (through November 8, 1991) will focus on understanding the basic mechanisms responsible for altering the membrane properties of lyophilized reconstituted human red cells. Our premise is that the stress of lyophilization and rehydration causes most cells to undergo a transient lysis-and-resealing. Such stress can induce alterations in several properties of red cell membranes: membrane permeability, membrane rigidity, membrane phospholipid composition and asymmetry, and membrane adhesion (cell-cell aggregation). Under Future Plans we will discuss the experimental approach to each of these potential problems. Identification of possible membrane damage will be our first priority, followed by buffer formulation research to minimize membrane damage or enhance rapid membrane re-annealing. Several new assays, including a cell filtration assay, will be used to complement our existing cell deformability assay to assess effects on the cell membrane. We plan to conduct a third series of clinical studies to measure the effects of such "repair" efforts, within the next six months.

On a longer term basis, our goal will be to identify conditions of cell processing that will minimize or avoid any identified membrane damage, in addition to use of "repair" solutions. Cryopharm's Process Development Group will examine key parameters for cell rehydration and cell freezing. We also plan to study residual moisture levels in dried cells, to evaluate whether excessive membrane dehydration contributes to observed loss of membrane flexibility. Subsequent clinical studies will be used to assess the impact of such improvements on cell survival.

In Section 3 of this report we include a draft manuscript entitled "Metabolic Activities of Freeze-Dried Human Erythrocytes". This paper is currently under scientific peer review for publication in the Proceedings of the National Academy of Sciences, U.S.A. The data demonstrate preservation of functional levels of both enzyme activities and metabolic intermediates of the glycolytic and pentose phosphate shunt pathways in lyophilized rehydrated human red blood cells. Measurable levels of methemoglobin repair are also demonstrated. We believe these data show for the first time that lyophilized red cells can be recovered as intact cells with functional energy metabolism. The ability of these cells to resume *de novo* synthesis of 2,3-diphosphoglycerate (2,3-DPG) and adenosine triphosphate (ATP) suggests that lyophilized red cells can be rehydrated with high preservation of oxygen-carrying function. Use of rejuvenation solutions with such rehydrated cells will be explored as part of a product development effort.

## RESEARCH REPORT

### Background

In the March 1991 progress report, we identified osmotic fragility of lyophilized human red cells as an important research milestone to achieve cell survival in vivo. This was based on our in vitro observations that lyophilized rehydrated red cells exhibited more lysis during incubation in physiological saline (only 28-32% of the reconstituted cells remained intact in saline, versus 98-100% for fresh control cells). In addition, the reconstituted cells exhibited a poor deformability response when subjected to mechanical shear and solution osmotic gradients (see Figure 1 in the March 1991 report). Autologous,  $^{51}\text{Cr}$ -labeled lyophilized red cells having these properties were infused into two normal volunteers during Cryopharm's first clinical study in October 1991 (these in vivo data were reported in our November 9, 1990 progress report). Our interpretation of the clinical data was that the fragile cells suffered from rapid intravascular hemolysis upon infusion. These results highlighted the need to improve the osmotic fragility and deformability of lyophilized red cells.

Cryopharm's basic red cell research developed an improved lyophilization and wash protocol that significantly improved the osmotic fragility and deformability profile of lyophilized cells (see Figure 3A and Appendix I in our March 1991 report). The lyophilized reconstituted cells used in the second clinical study, reported herein, exhibit an average 75-80% osmotic stability in saline. Cryopharm's objective for this second clinical study has been to determine the consequences of improved in vitro osmotic stability and deformability for in vivo cell survival.

### Clinical Study Methods

An updated clinical protocol is provided in Section 2 of this report. This protocol describes in detail the study procedures and the methods used for isotopic labeling of reconstituted cells with  $^{51}\text{Cr}$  sodium chromate, collection of urine and peripheral blood samples,  $^{51}\text{Cr}$  organ uptake, whole body gamma imaging, and calculation of whole blood volume and percent injected dose in circulation. Criteria used for volunteer selection and monitoring of vital signs during each study are also described.

### Experimental Results

#### A. In Vitro Measurements of Lyophilized Rehydrated Red Cells.

As part of the design of our clinical study, selected in vitro assays were performed on aliquots of each volunteer's lyophilized red cells immediately after rehydration and washing, and prior to infusion. The overall recovery of intact cells (as

measured by spun hematocrit), osmotic stability in saline, and standard red cell indices (using a hematology analyzer) were determined. Average cell density and degree of microscopic cell aggregation were also measured. These data are shown in Table 1 for the four volunteers used in this study (volunteers #4 - #7). Published ranges for these values derived from fresh red cells are shown for comparison.

The overall cell recovery ranged from 12-16% of the starting number of cells prior to lyophilization. These yields are reduced because we included a hypo-osmotic selection (cells were washed once in 250 mOsmol saline) after rehydration to eliminate weakened or osmotically unstable cells. Our rationale was to select a more homogeneous subpopulation of cells having a high average osmotic stability (79-81% in physiological saline), and to infuse these cells to determine their behavior in vivo. Our hypothesis was that this cell subpopulation having high osmotic stability and characterized by microcytic cells (mean cell volume, MCV, of about 73 cubic microns versus 80-100 for fresh cells) may exhibit measurable in vivo survival, assuming no other cell defects existed to compromise cell circulation.

For volunteers #6 and #7, an aliquot of rehydrated cells was returned by overnight delivery on wet ice to Cryopharm for determination of cell deformability by ektacytometry (Table 1). In these two samples the maximum cell deformability (DI max) was 54% and 66% of control fresh cells. The reduced DI max may correlate with the elevated cell aggregation observed in the samples.

Measurements of the prevalence of microscopic cells aggregates were conducted using a four point scoring system (1+ through 4+ as explained in Table 1). For fresh red cells, a low percent of the cells can be found in 2-4 cell aggregates, or "rouleux" (about 0.7% of the total cells counted are scored as 1+ aggregates, with no larger aggregates occurring). We observe a variable level of aggregation in lyophilized rehydrated cells, particularly of 2-4 cell clusters which can comprise 20-26% of the total cells. Although the sample prepared from volunteer #4 was not adequately rehydrated due to a minor container leak, comparable aggregation was observed with volunteers #6 and #7, in which the specified rehydration procedure was followed. We do not understand why less aggregation occurred in the sample from volunteer #5. Although each sample is passed through an in-line 20 micron blood filter during infusion, we believe that the observed cell aggregation points to an underlying cell membrane alteration that may explain the subsequent in vivo survival and organ uptake results (see Conclusions for further discussion).

## B. Results from Uptake Probe and Gamma Imaging.

Following isotopic labeling, each volunteer's own cells are infused according to standard procedures outlined in the clinical protocol. In Table 2 we show the subsequent organ distribution of the infused  $^{51}\text{Cr}$ , measured using an external uptake probe over marked areas of the body that designate the heart, liver, and spleen. To

monitor the dynamics of organ distribution, measurements were taken at 4 hours and at 24 hours post-infusion. These studies complement measurements of the level of  $^{51}\text{Cr}$  in peripheral circulation by revealing the apparent mechanism of cell clearance.

In Table 2 the raw percentages reflect the proportion of the total counts found over the heart, liver, and spleen as measured by the external uptake probe. As a check of the accuracy of these measurements, we also used gamma camera imaging for volunteers #6 and #7 (the corresponding percentages determined by gamma imaging are shown in bold type in Table 2). The values for both techniques appear to correlate (gamma imaging data for volunteer #7 at the 24 hour time point are not yet available).

The raw percentage uptake data are more easily expressed as dose ratios at the bottom of Table 2. For comparison, standard red cell sequestration studies using fresh  $^{51}\text{Cr}$  red cells from healthy persons show a 1:1:1 (heart : liver : spleen) ratio of distribution. The equal organ distribution for fresh cells reflects the equilibrium of freely circulating cells among three highly vascularized organs. In Cryopharm's first clinical study in October 1990 (volunteer 2), we observed the following organ distribution:

TIME POST- INFUSION	SPLEEN/ HEART	LIVER/ HEART	SPLEEN/ LIVER
4 hours	1.5	3.9	0.39
24 hours	2.4	5.4	0.45

We interpret these data from volunteer 2 as indicative of rapid intravascular hemolysis caused by the low osmotic stability (32%) of the rehydrated cells used in that study. Due to the low dose of cells, the free  $^{51}\text{Cr}$ -tagged hemoglobin released by cell lysis is rapidly bound to serum haptoglobin and then cleared via the normal pathway in the liver. Thus, in volunteer 2 we observe elevated liver/heart ratios (the heart value provides a baseline as red cells or free hemoglobin are not cleared by the heart). Some level of splenic sequestration is also evident, particularly by 24 hours post-infusion.

Compared to the above earlier data, we observe several differences in the current study (volunteers 4-7). The sample from volunteer #5 exhibited the least amount of cell aggregation *in vitro*, and at 4 hours post-infusion the liver/heart ratio approaches normal (liver/heart = 1.2). More early sequestration is seen in the spleen (spleen/heart = 1.9 and spleen/liver = 1.5 at 4 hours). We interpret these data as indicative of early splenic sequestration of intact cells by the splenic fenestrae and splenic macrophages. By 24 hours post-infusion, the  $^{51}\text{Cr}$  label in volunteer #5 begins to shift to the liver (liver/heart increases to 2.1, and spleen/heart decreases proportionately). We interpret this observation as indicative of cell degradation by the



splenic macrophages with subsequent recycling of  $^{51}\text{Cr}$  to the liver. The data from volunteer #6 corresponds well with that from volunteer #5.

In volunteers #4 and #7 (Table 2) we observe a prevalence of  $^{51}\text{Cr}$  clearance by the liver. Based on the high osmotic stability of these cells in vitro (81% and 79%), we do not believe that rapid intravascular hemolysis is a likely cause. Instead, we believe that the elevated cell aggregation observed in vitro may reflect an underlying membrane alteration that leads to formation of cell aggregates, especially when lyophilized cells are diluted into a large pool of fresh red cells. We suspect that intact cell aggregates were removed in these cases predominantly by the extensive microvasculature in the liver. As we will discuss in our conclusions, one hypothesis to guide our research invokes membrane damage that enhances cell "stickiness". An important research goal will be to understand the source of the observed variability between samples in the in vitro aggregation assay, and to explore other aggregation assays such as dilution of lyophilized cells into fresh autologous cells.

In Figure 1 we address the question of whether organ clearance of infused  $^{51}\text{Cr}$  red cells is evident in other microvascular beds such as in the lungs. Using gamma camera imaging, the spleen and liver of volunteer #6 are readily visible from a posterior aspect. From the anterior aspect, the heart and lungs can be seen to be clear, particularly when compared to the liver. (The gamma imaging count data shown in Table 2 are obtained by direct counting of the image within the boxed areas in Figure 1). Although the sample of lyophilized cells from this volunteer exhibited elevated levels of 2-4 cell aggregates (20% of the cells rated a 1+ visual score), little sequestration of the isotopic label appears in the lungs. Since the cells are infused into an arm vein, their first encounter with microvascular beds should occur in the lungs. We believe that the infused cells can initially survive in peripheral circulation, but are rapidly and selectively cleared in the microvasculature of the spleen and liver. This observation may implicate some autoantibody sensitization of the cells, which would render them susceptible to clearance by the splenic and hepatic reticuloendothelial (RES) systems. In this light, we note that the in vitro aggregation of certain lyophilized cell samples (notably volunteers #4 and #7), can interfere with standard clinical crossmatching based on hemagglutination. We therefore plan to use more direct assays to determine whether mild sensitization of the infused cells is occurring, which would contribute to their clearance via normal pathways for senescent red cells (Ref. 5).

### C. Percent Injected Dose in Circulation.

Given the rapid clearance of the infused cells, we believe that the uptake probe and gamma imaging data provide the best insight as to the possible underlying mechanisms. Monitoring of  $^{51}\text{Cr}$  levels in peripheral blood samples can be interpreted in light of the organ distribution observations. In Table 3 we show the level of  $^{51}\text{Cr}$  in

peripheral blood samples taken at various times post-infusion. Due to the rapid clearance of peripheral counts, we express these data as a percent of the injected dose, corrected for dilution in the whole blood volume as estimated from body weight and height (see clinical protocol), rather than using extrapolated time-zero. From Table 3 it is evident that most injected radiolabel is rapidly removed from peripheral blood in all four volunteers used in this study. In Graphs 1 and 2 these same data from the October 1990 study (Graph 1) can be compared to the present study (Graph 2). As we have discussed, in both studies peripheral cell circulation is rapidly diminished; however, based on the organ distribution we believe that fundamentally different mechanisms are responsible in each study (rapid intravascular hemolysis in the 1990 study versus organ sequestration of intact cells in the present study). In this light the peripheral blood counts complement the uptake probe data.

#### D. Percent of Injected Dose Excreted in Urine.

In any  $^{51}\text{Cr}$  red cell sequestration study, elution of free  $^{51}\text{Cr}$  from hemoglobin occurs at a reproducible rate (about 1% per day; Ref. 1). Free  $^{51}\text{Cr}$  is rapidly filtered by the kidneys and excreted in the urine (Ref. 2). In addition, release of free  $^{51}\text{Cr}$ -tagged hemoglobin by intravascular hemolysis can also lead to hemoglobinuria if the amount of hemoglobin saturates the level of serum haptoglobin (we do not infuse enough labeled red cells to saturate haptoglobin, even if 100% of the infused cells lyse immediately). As a standard control, we monitored urine excretion of the isotopic label. In Table 4 we show that loss of isotope in urine is relatively low (5-7% of the injected dose). We believe this reflects normal elution of Cr as well as possible release of Cr during splenic and hepatic phagocytosis or lysis of the infused red cells.

### Conclusions

From the experimental data we derive the following conclusions:

- 1) Infused lyophilized autologous red cells are still cleared rapidly from peripheral circulation despite improved overall osmotic stability and deformability profile, relative to our October 1990 clinical study. However, this clearance appears to reflect a different mechanism: sequestration of intact cells instead of immediate intravascular hemolysis.
- 2) Sequestration of the cells appears to predominantly involve the spleen and liver, relative to other microvascular beds (lungs).
- 3) Splenic and hepatic sequestration can reflect two mechanisms: immune-mediated clearance by the reticuloendothelial system, and/or microvascular entrapment of rigid cells or cell aggregates by the hepatic microvasculature and especially the splenic fenestrae.

In light of these conclusions, it is evident that the improved osmotic stability of our lyophilized cells was successful in overcoming a significant research hurdle. The remaining hurdle to an initial demonstration of cell survival appears to involve a syndrome of cell membrane damage or alterations that need to be identified in better detail in order for improvements to be achieved. In the next section on Future Research Plans, we will discuss several formal possibilities concerning the types of membrane alterations that could occur during lyophilization and rehydration, and discuss our proposed experimental approach to defining and resolving the problem.

## **FUTURE RESEARCH PLANS**

### **A. Identification of Potential Membrane Alterations in Lyophilized Rehydrated Cells and Approaches to Repair or Avoid Identified Changes.**

Following our hypothesis that cell membrane alterations are fundamental to the observed sequestration of lyophilized red cells, our basic research will focus first on identifying and understanding the underlying mechanisms. We believe there are several formal possibilities that need to be explored:

- 1) Membrane phospholipid asymmetry
- 2) Membrane phospholipid composition
- 3) Cell aggregation
- 4) Cell deformability (membrane rigidity)
- 5) Cell permeability and volume control
- 6) Antibody sensitization.

#### Studies on Membrane Phospholipids

Rehydration of lyophilized red cells using our existing conditions results in significant cell lysis (at least 50% of the starting cells are lost at rehydration). Hypotonic or hypertonic lysis of red cells can disrupt the normal asymmetric distribution of phospholipids between the inner and outer leaflets of the membrane bilayer. Loss of phospholipid asymmetry can lead to enhanced interactions between the damaged cells and phagocytic cells of the reticuloendothelial system. If such alterations in the membrane lipid packing are occurring, we believe that shear stress or osmotic stress during rehydration could disrupt the membrane. Excessive membrane dehydration may also contribute to such damage.

We propose to study the membrane phospholipid packing in lyophilized rehydrated cells using two approaches: thin layer chromatography (TLC) to evaluate membrane lipid composition (Ref. 6) and merocyanine 540 dye binding to evaluate the lipid distribution and packing in the outer membrane leaflet (Ref. 7). In both assays we will use fresh red cells as normal controls. In addition, we have conducted

preliminary studies with oxidizing reagents (phenazine methosulfate and t-butyl hydroperoxide) in concentrations demonstrated to cause perturbations of the lipid bilayer in fresh red cells. We will use these "treated controls" as positive measures of the effectiveness of the assays.

Merocyanine 540 is a cell-impermeant, fluorescent lipophilic dye. Merocyanine preferentially intercalates into membrane bilayers comprised of loosely packed lipids (Ref. 7). Membrane-damaged cells, such as cells treated with t-butyl hydroperoxide, bind significantly more fluorescent probe than normal cells. External application of this dye to red cells has been used to assess translocation of phospholipids in the outer membrane leaflet. We plan to conduct similar studies to compare the dye binding properties of lyophilized rehydrated cells versus fresh cells and treated controls.

Depending upon the nature of any identified perturbation to membrane phospholipid composition or asymmetry, we will initially try to repair any damage by incorporating membrane resealing agents (free phospholipids, salts, sterols) into the various buffers used to process the cells. We do not expect that this approach will solve such an issue, and therefore longer term effort will involve studies of residual moisture content in dried cells, to assess whether membrane dehydration contributes to dislocation of membrane lipids. In such a case a compromise may be reached between storage life and higher product residual moisture. Inclusion of agents that bind water in our lyophilization medium will also be explored.

#### Studies on Cell Aggregation

In the present clinical study we observed variable levels of cell aggregation in the lyophilized rehydrated samples prior to infusion. In addition, we have also conducted a preliminary study in which we seeded lyophilized rehydrated cells back into autologous packed red cells in 1:1 and 1:4 dilutions, to mimic initial infusion conditions. We have observed a tendency for lyophilized cells to aggregate with autologous fresh cells, as well as the aggregation observed in lyophilized samples alone (Table 1).

To study this effect we will use a light transmission method (Ref. 8) to study the kinetics of cell aggregation under these various conditions. Increased cell stickiness may prove to be a significant mechanism that underlies the observed rapid organ clearance of lyophilized cells *in vivo*. If this mechanism appears to be significant, we will explore the use of anti-aggregating agents on the behavior of our lyophilized cells. Certain polymeric compounds can be incorporated into the lyophilization or rehydration solutions to minimize cell-cell interactions, perhaps by "coating" the rehydrated cells. The ultimate efficacy of such an approach will require an *in vivo* evaluation, but in the short term we can study mixtures of lyophilized and fresh autologous cells.

### Studies on Cell Deformability (Filterability)

Our current lyophilization protocol has increased the mean maximum deformability of lyophilized red cells measured by ektacytometry (DI max) from 39% of the fresh cell value to 50-70% of the fresh cell value. In addition, the osmotic gradient profile (osmoscan) of our cells has also been significantly improved since October 1990. To complement these measurements, we will use a published cell filtration assay to evaluate the ability of lyophilized red cells to pass through a 5 micron pore filter, to mimic conditions encountered in the microvasculature (Ref. 9). This assay will use a cell transit time analyzer (essentially a conductivity meter) to measure the average time needed for red cells to traverse the filter. This system has been used to evaluate the filterability of red cells from sickle cell patients (Ref. 9). We expect that lyophilized rehydrated red cells will exhibit an abnormally high filter transit time, relative to fresh cells, reflecting their elevated membrane rigidity.

We will use the cell filtration assay to measure the ability of fresh versus lyophilized red cells to traverse the narrow filter pores. Red cells damaged by treatments with oxidizing agents can also be used as assay standards. The importance of this assay will be as a further method to evaluate and monitor improvements to the lyophilization and rehydration conditions.

We believe that polymers that may find usefulness in blocking cell aggregation may also improve the ability of lyophilized red cells to traverse filter pores. Again, the ultimate efficacy of the approach will require an *in vivo* evaluation. On a longer term basis, we believe that more defined freezing conditions, cryoprotectant loading, and rehydration conditions will likely impact a number of cell membrane properties.

### Studies on Membrane Permeability

Based on the reduced MCV and MCH (Table 1) observed in our lyophilized rehydrated cells, we believe that these cells undergo a transient lysis-resealing during rehydration. The resulting microcytic cells retain their discocytic morphology, but may still be leaky as a consequence of inefficient membrane resealing. One method to monitor membrane leakiness uses carboxyfluorescein to detect loss of membrane integrity (the fluorescent dye is normally excluded from cells, but can be incorporated into leaky cells). We expect to detect some level of altered membrane permeability, and will use this assay to evaluate process improvements.

To address this issue we plan to evaluate membrane resealing agents in our buffer solutions, to promote more rapid and efficient resealing. For example, certain salts have been used to promote membrane resealing (Ref. 10). Second, we plan to study the rehydration conditions, which we believe cause the most mechanical stress to the cell membranes and therefore promote leakiness and loss of membrane integrity. Use of different solute concentrations in the rehydration solution will be explored to try to minimize cell swelling due to the rapid entry of water in the dried cells. Our

rationale will be to slow the rate at which water enters the cell, in order to allow more time for each cell to export internalized cryoprotectant before reaching a critical cell volume.

### Studies on Antibody Sensitization

It remains a formal possibility that the observed splenic and hepatic sequestration of lyophilized red cells is due in part to immune clearance mediated by binding of autoantibodies to the altered red cell surface. Although we have not detected clear evidence of antibody sensitization using clinical crossmatching, more sensitive assays are available that do not rely on simple hemagglutination to detect a reaction (Ref. 11-12). We plan to conduct studies using radiolabeled anti-human second antibodies to directly address this issue. If such autoantibody binding is detected, then presumably our cells appear similar to senescent red cells when infused (Ref. 11-12). This assay may prove to be a useful tool to evaluating the effects of lyophilization on the outer membrane surface. We believe that if antibody sensitization is occurring, that the approaches already described to resolve other issues of membrane damage may alleviate such immune recognition.

### REFERENCES

- 1) ICSH Recommended Method for Radioisotope Red Cell Survival Studies (1980). Br. J. Haematol. 45, 659-666.
- 2) Schloesser, L.L., et al. (1957). J. Clin. Invest. 36, 1470-1485.
- 3) Spencer, R.P. (1980). J. Nucl. Med. 21, 489-491.
- 4) Anderson, D.R. and Kelton, J.G. (1989). In: "Immune Destruction of Red Blood Cells", S.J. Nance, ed. American Association of Blood Banks, Arlington, VA.
- 5) Kay, J. (1981). Nature 289, 491.
- 6) Skipski et al. (1964). Biochem. J. 90, 374-377.
- 7) Schlegel et al. (1980). Cell 20, 321-328.
- 8) Sowemimo-Coker, S. (1989). Biorheology 26, 951-972.
- 9) Sowemimo-Coker, S. et al. (1990). Blood 76, 296.
- 10) Hurel et al. (1987). Advances in the Blood Sciences 67, 37-46.

11) Kay, J. (1978). *J. Supramol. Struc.* **9**, 555.

12) Kay, J. (1981). *Nature* **289**, 491.

**TABLE 1**  
**IN VITRO CELL INDICES**

	<u>Fresh RBC's</u>	<u>Study #4</u>	<u>Study #5</u>	<u>Study #6</u>	<u>Study #7</u>
Overall Recovery		16.20%	12%	13.10%	15.80%
Osmotic Stability	98-100%	81.20%	81.30%	79.20%	79.30%
MCV (Cu Microns)	80-100	84.7	73.6	73.6	79.3
MCH (pg)	25-35	29.3	20.5	21.4	24.9
MCHC (%)	31-37	34.6	27.9	29.1	31.4
DI Max *	0.672	ND	ND	0.353	0.441
% Control *	100%	ND	ND	54.20%	66.10%
Aggregation					
% 1+	0.7	25.1	6.4	20	25.9
% 2+	0	0.004	0.18	0.53	0.97
% 3+	0	0	0	0	0.32
%4+	0	0	0	0	0
Density	1.090-1.100	1.080	1.086	1.084	1.081

Samples Analyzed at Cryopharm 24 Hours Post-Transfusion

**Aggregation:**

1+ = 2, 3 or 4 cells

2+ = 5, 6 or 7 cells

3+ = 8, 9 Or 10 cells

4+ = > 10 cells



**TABLE 2**  
**RESULTS FROM UPTAKE PROBE**  
**AND GAMMA IMAGING**

	<u>Heart</u>	<u>Liver</u>	<u>Spleen</u>
<b>Study #4:</b>			
4 Hour	10.0%	50.0%	40.0%
24 Hour	8.2%	60.0%	31.8%
<b>Study #5:</b>			
4 Hours	24.3%	30.3%	45.4%
24 Hours	21.4%	44.3%	34.4%
<b>Study #6:</b>			
4 Hours	22.5% <b>(22.2)</b>	38.2% <b>(37.4)</b>	39.4% <b>(40.3)</b>
24 Hours	21.2% <b>(18.8)</b>	42.2% <b>(39.7)</b>	36.6% <b>(41.6)</b>
<b>Study #7:</b>			
4 Hours	22.4% <b>(26.8)</b>	58.1% <b>(57.6)</b>	20.0% <b>(15.5)</b>
24 Hours	20.4%	61.7%	17.9%

**Text:** = Gamma Image Percentages

Text = Uptake Probe Percentages

**DOSE RATIOS:**

	<u>SPLEEN/HEART</u>	<u>LIVER/HEART</u>	<u>SPLEEN/LIVER</u>
<b>Study #4:</b>			
4 Hours	4.0	5.0	0.8
24 Hours	3.9	7.3	0.5
<b>Study #5:</b>			
4 Hours	1.9	1.2	1.5
24 Hours	1.6	2.1	0.8
<b>Study #6:</b>			
4 Hours	1.7	1.7	1.0
24 Hours	1.7	2.0	0.9
<b>Study #7:</b>			
4 Hours	1.14	3.0	0.39
24 Hours	1.14	3.5	0.33

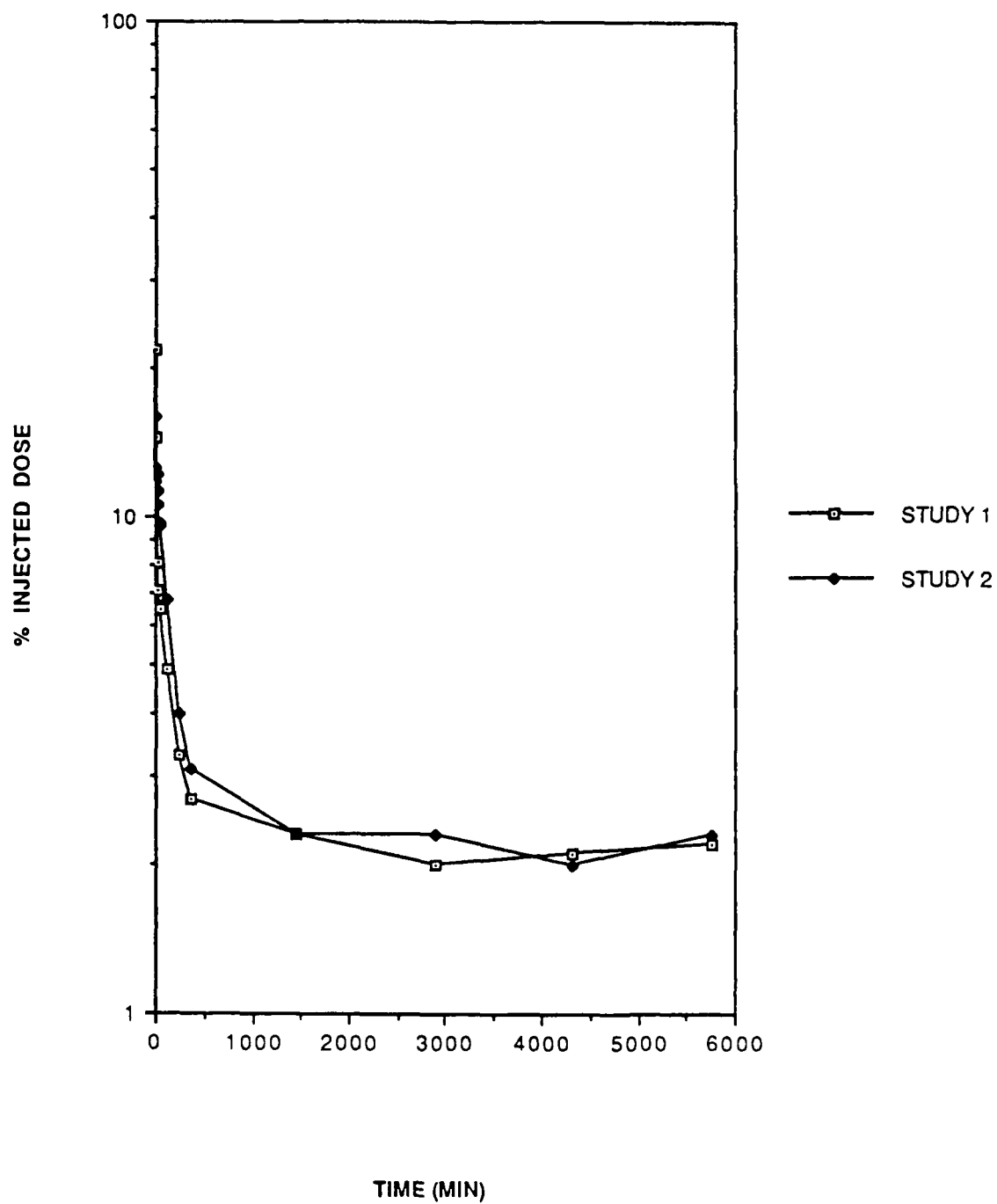
**TABLE 3**  
**% INJECTED DOSE**

Time Point	Study #4	Study #5	Study #6	Study #7
5 min	24.0	41.5	29.5	15.7
7.5 min	12.8	24.5	19.5	10.9
12 min	7.6	Not done	12.7	7.6
15 min	6.8	12.5	9.53	6.9
20 min	6.1	8.0	8.50	6.3
30 min	5.1	5.7	7.85	4.7
45 min	4.3	4.6	6.68	4.3
60 min	4.1	3.9	6.17	3.8
2 hr	1.8	3.1	4.50	3.0
4 hr	Not done	2.5	3.61	2.2
6 hr	1.5	2.1	3.24	Pending results
24 hr	1.2	1.7	2.29	
48 hr	0.9	Not done	1.90	
72 hr	0.8	1.4	1.73	
96 hr	0.8	1.3	Pending result	✓

**TABLE 4****% INJECTED DOSE EXCRETED IN URINE**

<b>Time Point</b>	<b>Study #4</b>	<b>Study #5</b>	<b>Study #6</b>
0 - 6 Hours	595,200 cpm	291,200 cpm	417,900 cpm
6 - 12 Hours	339,475 cpm	321,360 cpm	453,849 cpm
12 - 18 Hours	246,480 cpm	156,600 cpm	0 cpm
18 - 24 Hours	221,125 cpm	97,920 cpm	156,552 cpm
% Injected Dose Excreted in Urine	7.0 %	5.2 %	7.0%

GRAPH 1  
% INJECTED DOSE STUDY 1 & 2



GRAPH 2  
% INJECTED DOSE STUDY 4,5,6,7

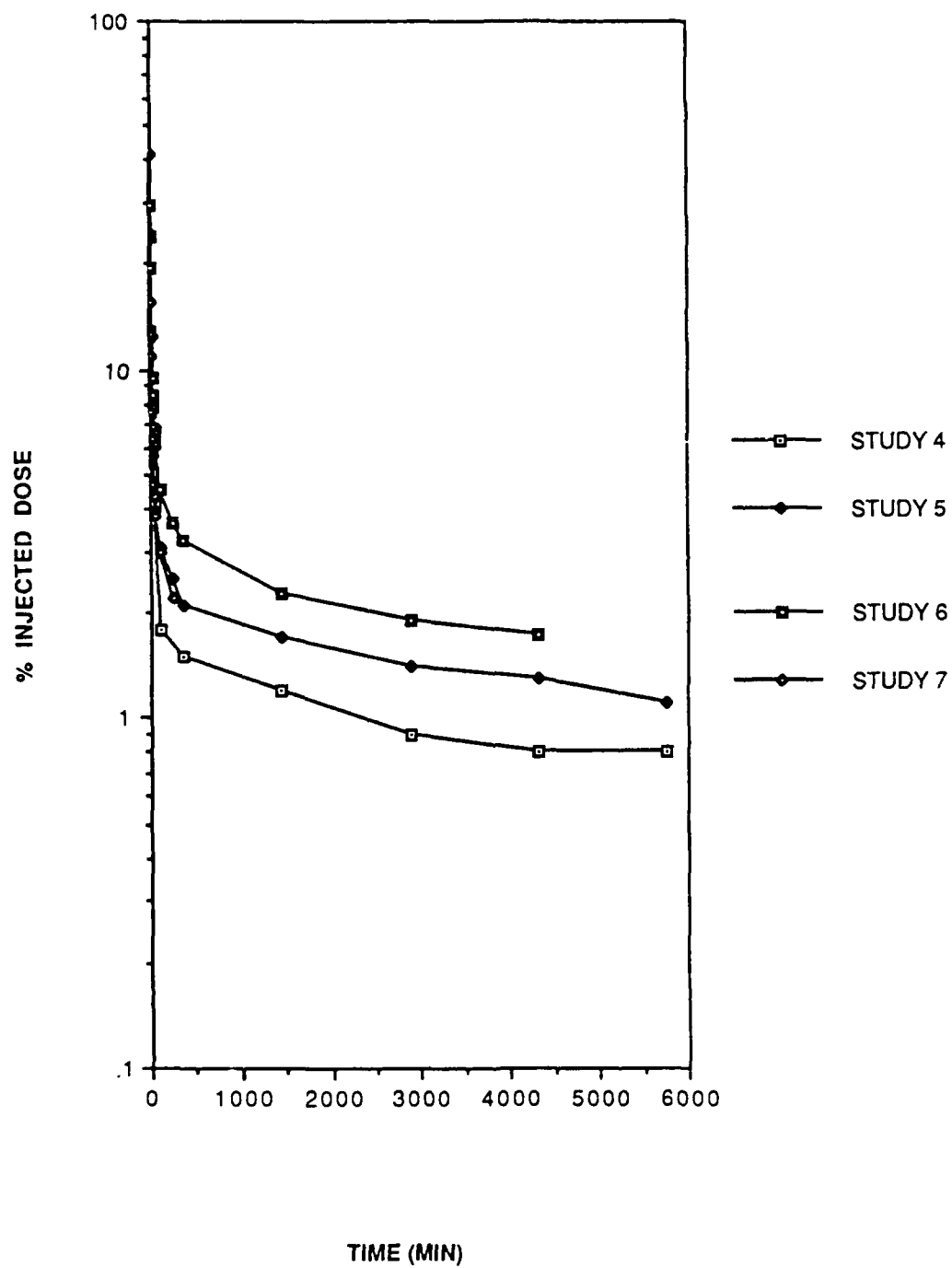
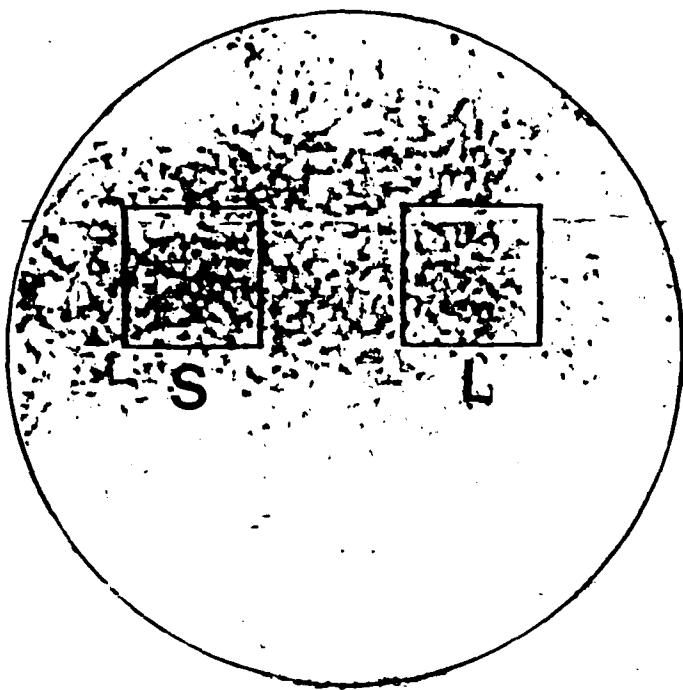
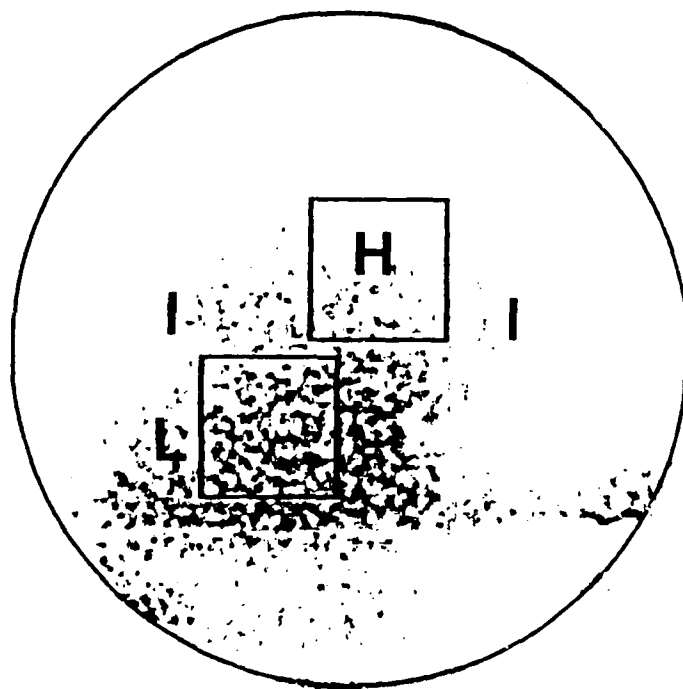


FIGURE 1

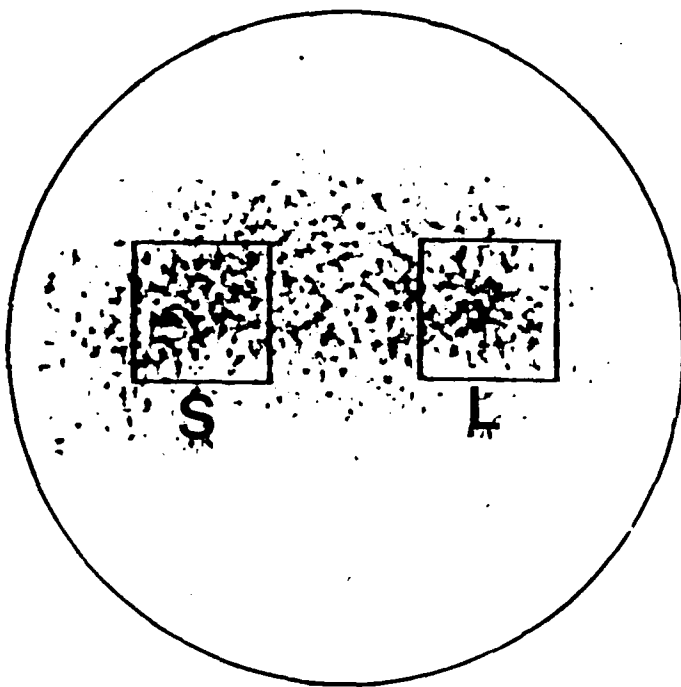
GAMMA IMAGE RESULTS FROM STUDY #6



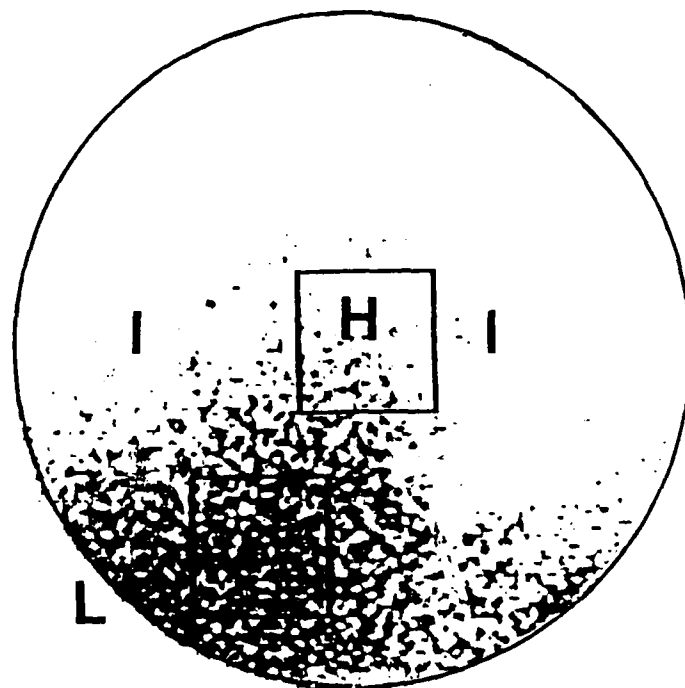
4 Hour Posterior



4 Hour Anterior



24 Hour Posterior



24 Hour Anterior

H = Heart

S = Spleen

L = Liver

I = Lung

**CRYOPHARM CORPORATION**

**PROTOCOL**

**A CLINICAL EVALUATION OF THE SURVIVAL OF AUTOLOGOUS  
LYOPHILIZED AND RECONSTITUTED 51-CHROMIUM LABELED  
HUMAN RED BLOOD CELLS IN VIVO**

May 1991

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## 1.0 INTRODUCTION

Cryopharm Corporation has developed a novel freeze-drying process for lyophilizing and reconstituting human red blood cells. The company has evidence that closely related primate red blood cells (macaque or baboon) will not serve as adequate models for making useful improvements in this process, and is therefore conducting this study of very small doses in humans so that further process improvements can be investigated.

Cryopharm has conducted several "dry runs" in which blood was collected, shipped, processed, shipped again, reconstituted, and labeled with 51-Cr. These dry runs were successfully conducted to confirm the logistics and sterility of the procedures used in this study.

## 2.0 OBJECTIVES

The objective of this study is to determine the in vivo survival of 51-Cr labeled lyophilized and reconstituted human red blood cells.

## 3.0 STUDY DESIGN AND DESCRIPTION

This single-center study will test the in vivo survival of lyophilized, reconstituted red blood cells in twelve to fifteen healthy male volunteers. All transfusions will be performed with autologous blood samples and the study will proceed one subject at a time to minimize cross contamination risks. Eligible study subjects will be phlebotomized one unit (450 ml) of whole blood at the blood donor center of the study site. This blood will be shipped to Cryopharm where it will be washed and the packed red cells will then be lyophilized, and then shipped back to the study site. The lyophilized red cells will be stored refrigerated until use. Fourteen days after phlebotomy, the subject will return to the study site, at which time a 5-25 ml aliquot of reconstituted 51-Cr labeled autologous red blood cells will be infused intravenously into a large arm vein. The subject will remain in confinement at the study site for 24 hours following the transfusion and return on the following four days (Study Days 2 through 5) to have peripheral blood samples collected for radioactivity counting. The subject will return seven days post-infusion (Study Day 8) for follow-up examination and procedures.

## SUBJECT SELECTION

To be eligible for participation in the study, subjects must meet the following criteria prior to having the blood sample for lyophilization taken:

1. Male, age 21 to 35 years, inclusive.
2. Medical history, vital signs, physical examination including fundoscopic and neurologic examinations, and laboratory tests (Section 5.1.7) without evidence of clinically significant medical condition, in particular, no evidence of hepatitis.
3. A 12-lead electrocardiogram (ECG) without clinically significant abnormality.
4. Weight within the following limits for height:

Height Without Shoes (cm)	Weight Indoor Clothing Without Shoes (kg)
156-158	51-77
159-161	52-97
162-163	54-81
164-166	55-83
167-168	56-85
169-170	57-88
171-173	58-90
174-176	59-93
177-178	60-95
179-181	61-98
182-184	62-100
185-186	63-103
187-189	64-106
190-191	65-109
192-193	67-111

-----

5. No known drug hypersensitivity, atopy or known seasonal or other allergy.
6. Subject does not take any medication on a chronic basis and has not taken any medication including over the counter medication and alcohol within the previous week. In addition, subject has not received any medication with known liver or kidney toxicity in the previous six months.

7. Subject does not have a history of:
  - Renal disorders or BUN, creatinine, uric acid, sodium, potassium or chloride values outside the investigator's normal range at any pretransfusion evaluation.
  - Hepatic disorder or AST, ALT, GGT, LDH, total bilirubin or direct bilirubin values outside the investigator's normal range at any pretransfusion evaluation.
  - Bleeding/coagulation disorder or severe anemia.
8. Negative urine drug and blood alcohol screens.
9. Subject is HIV antibody negative.
10. Subject has voluntarily signed the St. Elizabeth's Hospital of Boston Informed Consent Form after the nature of the study has been explained. Subject has also reviewed Appendix I of this protocol.

## 5.0 STUDY PROCEDURES

### 5.1 SCREENING PROCEDURES

Seven days prior to phlebotomy (Study Day -21), the following will be obtained to assess compliance with the subject selection criteria outlined in Section 4.0:

1. Informed consent documented in writing.
2. Medical history.
3. Height and weight.
4. Vital signs: sitting blood pressure and pulse, respiratory rate, and temperature.
5. Complete physical examination.
6. 12-lead electrocardiogram.
7. Laboratory Tests: All blood and urine samples will be collected and handled in accordance with accepted laboratory procedures. Blood samples for determination of prothrombin time and activated partial thromboplastin time will be collected after the samples for hematology, and blood chemistry.

Obtain blood and urine samples after at least an eight-hour fast for the following laboratory tests:

Hematology: hemoglobin, hematocrit, red blood cell count, white blood cell count with differential, and platelet count.

Blood Chemistry: blood urea nitrogen (BUN), creatinine, aspartate aminotransferase (AST/SGOT), alanine aminotransferase (ALT/SGPT), gamma glutamyl transferase (GGT), lactate dehydrogenase (LDH), alkaline phosphatase, total bilirubin, direct bilirubin, total protein, albumin, uric acid, calcium, inorganic phosphorus, glucose, sodium, potassium, chloride, bicarbonate.

Coagulation: prothrombin time (subject and control) and activated partial thromboplastin time (subject and control).

Urinalysis: pH, specific gravity, albumin, blood, glucose, and microscopic examination to include white blood cells, red blood cells, bacteria, casts, and crystals.

## 5.2 DAY OF PHLEBOTOMY (STUDY DAY -14)

The following will be obtained fourteen days prior to transfusion with radioactively labeled RBCs:

1. Medical history update.
2. Vital signs, as described in Section 5.1.4.
3. Hematocrit, blood alcohol screen, and urine drug screen.
4. Blood collection: One unit of whole blood (450 ml) will be collected by a trained phlebotomist in a standard blood collection bag containing CPD. Subjects will be screened by the phlebotomist according to standard screening procedures for voluntary blood donation. The packed red blood cells then will be shipped at 4°C to Cryopharm (next day delivery). All blood products collected at this time will be subjected to the usual screening procedures (ALT, HIV HB<sub>s</sub>A<sub>g</sub>) for voluntarily donated blood products.

Each sample will be processed by Cryopharm (as described in Appendix II) and shipped back to the study site within ten days. Upon arrival, the sealed bag should be kept refrigerated prior to reconstitution.

**5.3 MORNING OF TRANSFUSION (STUDY DAY 1)**

Subjects will report to the study site on the morning of transfusion when the following will be performed to verify compliance with the subject selection criteria. All results must be available before transfusion.

1. Medical history update.
2. Weight: Each subject will be weighed just before transfusion of labeled cells so that total blood volume can be estimated.
3. Vital signs, as described in Section 5.1.4.
4. Physical examination.
5. Laboratory tests, as described in Section 5.1.7. Be sure to obtain the subject's hematocrit at this time.
6. Urine drug and blood alcohol screen.

**5.4 TRANSFUSION PROTOCOL**

Following screening procedures, subjects will be admitted to the clinical research unit of the study site as inpatients. They will remain as inpatients for approximately 24 hours following transfusion of autologous blood.

- 5.4.1 Reconstitution of the RBCs:** Lyophilized RBCs from the study subject will be reconstituted at the study site using a Cobe cell washer according to the Cryopharm protocol (refer to Appendix III). The reconstituted red blood cells will be removed from the Cobe bag through a blood filter using sterile technique and placed in a sterile glass vial. The vial will be centrifuged to obtain packed red blood cells for labeling. The supernatant will be removed and two aliquots (3-5 ml each) will be used to inoculate blood culture bottles. A sample of the red cells will be sent to the blood bank for type and crossmatch.

- 5.4.2 Chromium Labeling of Red Blood Cells: The vial containing the packed reconstituted red blood cells will be brought to the Nuclear Medicine Department. The 5-25 ml of red cells will be incubated for 30 minutes at room temperature with 150-200 microcuries of sterile 51-Cr by standard technique. After quenching with sterile ascorbic acid, the red cells are ready for transfusion. A detailed labeling protocol is included in Appendix III.
- 5.4.3 Transfusion of Labeled Red Blood Cells: An indwelling catheter with a heparin lock will be inserted into a large vein in the subject's forearm. The labeled autologous red blood cells will be infused through a scalp vein into the contralateral arm through a 19 gauge butterfly set.
- 5.4.4 Vital Signs will be measured hourly following transfusion for four hours, then once per shift until hospital discharge, as described in Section 5.1.4.
- 5.4.5 Blood Sampling for Red Blood Cells Survival: Following administration of labeled cells ( $t = 0$ ), peripheral blood samples (5 ml each) will be collected. Five ml samples of whole blood will be collected into purple stopper (EDTA) blood collection tubes through the indwelling heparin lock. An initial portion will be withdrawn and discarded. The indwelling catheter will be flushed with normal saline following each blood collection.

The whole blood aliquots will be collected at the following times post-transfusion:

<u>Post-transfusion Blood Collection Times</u>					
<u>Time</u>	<u>Day 1</u>	<u>Day 2</u>	<u>Day 3</u>	<u>Day 4</u>	<u>Day 5</u>
0		X	X	X	X
5 minutes	X				
7.5 minutes	X				
12 minutes	X				
15 minutes	X				
20 minutes	X				
30 minutes	X				
45 minutes	X				
1 hour	X				
2 hours	X				
4 hours	X				
6 hours	X				

The collection of blood will take precedence over all other study activities. Numerous samples are needed in the first hour and several during Day 1 as damaged red cells are very rapidly cleared from circulation. Provided enough

radioactivity remains in circulation, the study will collect samples through Day 5. The time that each blood sample is drawn will be recorded to the nearest minute on the appropriate case report form for each subject.

Each 5 mL sample of whole peripheral blood collected in EDTA will be counted in the Nuclear Medicine Department according to their standard protocol. The hematocrit will be determined for each blood sample withdrawn. (see Appendix III).

5.4.6 **Urine Collection:** Subjects will be instructed to void and empty their bladders completely just prior to transfusion. Following transfusion, subjects will void into a bedside urinal. Urine volume will be recorded and urine will be pooled into a large plastic bottle at six hourly intervals for gamma counting. The percentage of injected dose excreted in urine will be calculated.

5.4.7 **Gamma Imaging and External Counts:** Subjects will be taken to Nuclear Medicine for gamma camera imaging and external counts 4-hours and 24-hours after transfusion of labeled red blood cells. Gamma imaging will be taken using posterior and anterior views. External counts will be taken over the precordium, liver and spleen with the following ratios determined:

liver/precordium  
spleen/precordium  
spleen/liver

## 5.5 FOLLOW-UP EVALUATIONS (STUDY DAY 8)

The following procedures and evaluations will be performed seven days post-transfusion.

1. Medical history update.
2. Vital signs, as described in Section 5.1.4.
3. Physical examination.
4. Hematology and blood chemistry laboratory tests, as described in Section 5.1.7.

## 6.0 MANAGEMENT OF INTERCURRENT EVENTS

### 6.1 DIETARY RESTRICTIONS

Subjects will be instructed not to consume any alcohol-containing beverage during the period from seven days prior to phlebotomy to the completion of the follow-up evaluations.

6.2 CONCURRENT MEDICATION

No medication, including over-the-counter medication, is to be administered to or taken by any subject from two weeks prior to autologous blood transfusion until after study completion. If a subject reports taking any medication, a medical monitor at Cryopharm must be notified immediately and the incident must be documented on the appropriate case report form.

6.3 ACTIVITY RESTRICTIONS

No vigorous exercise will be permitted from Day -14 until after study completion.

6.4 ADVERSE EVENTS

Throughout the duration of the study and especially for the first 12 hours, the investigator will closely monitor each subject for evidence of transfusion intolerance and for the development of clinical or laboratory evidence of an adverse event or any signs of hypersensitivity reaction. All adverse events which occur during the course of the study must be reported in detail on the appropriate case report form and followed to a satisfactory resolution.

The description of the adverse event will include the date, time of onset, duration, severity, etiology, the relationship of the adverse event to the Investigational lyophilized reconstituted red cells and any treatment required.

If a serious or unexpected adverse event occurs, and/or if a subject dies from any cause during or within one month after investigational red cell administration, whether related to the study or not, one of the following monitors must be notified within 24 hours by telephone:

Christine Williams  
Cryopharm Corp.  
(818) 793-1040

Roger Hackett  
Cryopharm Corp.  
(818) 793-1040

A written confirmation of the serious or unexpected adverse event or death, including autopsy report, if available, will be sent to a Cryopharm monitor within five days of the telephone report.

The investigator will rate the severity of any adverse event according to the following definitions:

Mild: The adverse event is transient and easily tolerated by the subject.



Moderate: The adverse event causes the subject discomfort and interrupts the subject's usual activities.

Severe: The adverse event causes considerable interference with the subject's usual activities, and may be incapacitating or life-threatening.

The investigator will use the following definitions to assess the relationship of the adverse event to the investigational red cells:

Probable: The adverse event has a timely relationship to transfusion and a potential alternative etiology is not apparent.

Possible: The adverse event has a timely relationship to transfusion. However, a potential alternative etiology exists which may be responsible for the adverse event.

No relationship: Definite evidence exists that the adverse event is related to an etiology other than transfusion. The etiology must be stated on the case report form.

#### 6.5 **PREMATURE DISCONTINUATIONS**

Each subject has the right to withdraw from the study at any time without prejudice. The investigator may discontinue any subject's participation when he feels it is necessary for any reason, including adverse event or failure to comply with the protocol.

Should a subject withdraw from the study, the reason(s) must be stated on the case report, and the following evaluations of the subject should be performed: medical history update, vital signs, complete physical examination, ophthalmologic examination, neurologic assessment, ECG, EEG, laboratory tests (hematology, coagulation, blood chemistry, and urinalysis), and collection of archive samples.

#### 6.6 **MODIFICATION OF PROTOCOL**

Neither the investigator nor the Cryopharm monitor will modify this protocol without first obtaining concurrence of the other. The modification must be documented in writing. Any change in the research activity, except that necessary to remove an apparent immediate hazard to the subject, must be reviewed and approved by the St. Elizabeth's Hospital Institutional Review Board prior to implementation. Cryopharm may submit protocol amendments which may be subject to the St. Elizabeth's Hospital Institutional Review Board approval.

#### **6.7 DEPARTURE OF PROTOCOL FOR AN INDIVIDUAL SUBJECT**

When a significant departure from the protocol is deemed necessary for an individual subject, the investigator or other physician in attendance must contact the Cryopharm monitor.

Such contact with the Cryopharm monitor will be made as soon as possible to permit a decision as to whether or not the subject is to continue in the study. Any departure from the protocol will be authorized only for that one subject. A description of the departure from the protocol and the reason(s) for it will be recorded on the appropriate case report form.

#### **7.0 CASE REPORT FORMS**

Case report forms are provided for each subject. Each form must be filled out completely and legibly in black ink. Corrections of data on the case report form must only be made by crossing out the incorrect values and writing the correct values next to those crossed out. Each correction must be initialed by the investigator or an authorized assistant.

#### **8.0 INSTITUTIONAL REVIEW**

Approval must be obtained from the St. Elizabeth's Hospital of Boston Institutional Review Board (IRB) prior to participation of human subjects in these research studies. Confirmation of protocol and informed consent approval and a list of members of the Review Board and their qualifications and affiliations will be provided to Cryopharm prior to the onset of the study.

#### **9.0 SUBJECT CONFIDENTIALITY**

All reports and communications relating to subjects in the study will identify each subject only by the subject's initials and by the subject's study number. The investigator agrees to furnish Cryopharm with complete subject identification on the confidential follow-up form, which will be used for purposes of long-term follow-up if needed. This will be treated with strict adherence to professional standards of confidentiality, and will be filed at Cryopharm under adequate security.

#### **10.0 USE OF INFORMATION AND PUBLICATION**

All information concerning the blood lyophilization process and Cryopharm operations, such as Cryopharm's patent applications, formulas, manufacturing processes, basic scientific data, or formulation information, supplied by Cryopharm and not previously published is considered confidential information.

The information developed during the conduct of this clinical study is also considered confidential and will be used by

Cryopharm in connection with the development of the red blood cell lyophilization process. This information may be disclosed as deemed necessary by Cryopharm. To allow for the use of the information derived from this clinical study and to insure complete and thorough analysis, the investigator is obligated to provide Cryopharm with complete test results and all data developed in this study.

This confidential information shall remain the sole property of Cryopharm, shall not be disclosed to others without the written consent of Cryopharm, and shall not be used except in the performance of this study.

Should the investigator choose to publish the results of this study, a copy of the manuscript will be provided to Cryopharm at least 40 days prior to the date of submission to the intended publisher. In the event Cryopharm chooses to publish the data from this study, a copy will be provided to the investigator at least 40 days prior to the date of submission to the intended publisher.

#### 11.0

#### INVESTIGATOR'S AGREEMENT

I have received and reviewed the Executive Summary, "Information for Clinical Investigator", for Cryopharm's red blood cell lyophilization process.

I have read the protocol and agree to:

A. Conduct the study as outlined herein

and

B. Maintain the confidentiality of all information received or developed in connection with this protocol.

\_\_\_\_\_  
Signature of  
Principal Investigator

\_\_\_\_\_  
Date

TABLE I  
STUDY SCHEMATIC

Evaluation/Procedure	Screening (Day-21)	Day -14	Day 1	Day 2	Day 3	Day 4	Day 5	Day 8 Follow-up
Medical History	x	x	x					x
Height & Weight	x		x <sup>+</sup>					
Vital Signs	x	x	x					x
Physical Examination	x		x					x
Endoscopic Exam	x							
ECG	x							x
Laboratory Tests	x	x	x					x
Urine Drug & Blood Alcohol Screen		x	x					
HIV Antibody Screen		x						
Written Informed Consent	x							
10-mL Blood Collection for Lyophilization		x						
Transfusion of Labeled RBCs			x					
5 mL Blood Collection for Radioactivity Counts			x <sup>+</sup>	x	x	x	x	
24-Hour Urine Collection for Radioactivity Count			x-----x					
Gamma Imaging and External Counts			x	x				

- \* Each subject will be weighed immediately prior to transfusion.  
 + Collected at 5, 7.5, 12, 15, 20, 30, 45 and 60 minutes and 2, 4, and 6 hours post-infusion.

## APPENDIX I

1. I understand that my eligibility to fully participate in the study will be determined, in part, by a medical history, physical examination, electrocardiogram, blood chemistry and hematology tests, and urine tests. The screening of these blood tests will require approximately two tablespoons of my blood. They will include a complete blood count, kidney and liver function tests, and tests to determine whether I have been exposed to hepatitis B virus and to HIV (AIDS).
2. I understand that if I remain eligible to participate in the study following screening, a unit (approximately 1 pint) of my blood will be withdrawn through a large vein in my arm. This process will be performed according to the procedures for voluntary blood donation at St. Elizabeth's Hospital. I understand that this procedure will include having my blood pressure and pulse rate determined, having my hematocrit (similar to blood count) determined, and filling out a questionnaire regarding my risk of exposure to the HIV (AIDS) virus. A brief medical history will also be obtained by the phlebotomist (person who draws my blood). At this point I understand that, although I am participating in a research project, this blood donation must be taken seriously. I will refrain from vigorous exercise once I have given the pint of blood and until my follow-up examination is completed. I understand that a sample of the blood collected from me will be screened for exposure to hepatitis or HIV according to standard blood donor procedures.
3. On the morning of transfusion of my re-hydrated freeze-dried red blood cells, I will report to the study site for an interim medical history and physical examination. Blood samples will be obtained (two tablespoons) for hematology and chemistry tests and a blood alcohol level. I will also provide a fresh urine specimen for drug screening. A small catheter will be inserted into a vein in my arm for the purpose of obtaining blood samples during the 24 hours. About two tablespoons of my freeze-dried and rehydrated blood cells will be made radioactive with <sup>51</sup> chromium and infused into my other arm through a hypodermic needle. No more than 200 microcuries of radioactive label will be used. This amounts to 2.4 millirads of radiation exposure, which is less than 40% of the amount of radiation from a chest x-ray. Blood samples (1 teaspoon each) will be obtained through the catheter in my arm 8 times during the first hour after transfusion, 2 hours after transfusion, 4 hours after transfusion and 6 hours after transfusion. This will be to determine how long the radioactive blood cells can be detected in my veins. I will be admitted to the hospital and remain in the hospital for 24 hours after the transfusion. During this time I will collect all of my urine in a plastic container as instructed by my nurse. Twenty four hours after the transfusion, another blood sample (1 teaspoon) will be obtained, the catheter will be removed from my arm, and I will be discharged from the hospital.

4. I will return to the study site daily for the following four days to have a blood sample (1 teaspoon each) withdrawn from an arm vein.
5. I will return to the study site for a follow-up history, physical examination and laboratory evaluation seven days after transfusion. At this time up to two tablespoons of blood will be drawn for blood chemistry and hematology testing.
6. I understand that I am not to consume any alcoholic beverage during the period from screening evaluation (19 days prior to transfusion) through completion of the follow-up evaluation (the 7th day following transfusion). In addition, I understand that I must not consume medication, including non-prescription medication, during this time. If I do take medication during the study I must promptly notify Dr. Weinstein at (617) 789-3000, X3081.
7. I understand that the procedures performed for the purpose of the study are being performed for clinical research, and will be performed at no cost to me. I will be compensated at the rate of \$50.00 per day for each active day of participation in the study, including screening evaluation, phlebotomy, five days of blood sampling after the transfusion, and follow-up visit. A bonus of \$100.00 will be paid for successfully completing the entire study. The total potential remuneration to me for participating in the study is \$500.00.
8. I understand that the cell circulation results obtained during the initial few hours following the injection of my radioactive red blood cells may not warrant continuation of the study. In this case I will remain in the hospital for the first 24 hours after injection of the radioactive cells, but I may not have to return on the following four days to provide a blood sample. I will return to the hospital for the scheduled follow-up examination. I understand that if the study is shortened in this manner, my potential total remuneration will remain \$500.00 provided I successfully complete the study through follow-up.

## APPENDIX II

### The Lyophilization of Red Blood Cells at Cryopharm

Each unit of packed red cells received from the St. Elizabeth's clinical coordinator will be inspected and released by Quality Control at Cryopharm prior to processing in the Company's certified class 100 clean room (A class 100 rating denotes fewer than 100 particles per cubic foot that are 0.5 micron or larger). The clean room is routinely disinfected and tested for viable microbials. The packed red blood cells will be processed using standard procedures to produce packed red blood cells. The packed cells will be washed using a Cobe Cell washer and sterile dextrose saline (commercially available). The washed cells and the sterile lyophilization buffer will be aseptically transferred into a pre-weighed lyophilization bag. Cryopharm's lyophilization blood bags are manufactured from approved blood bag materials and are certified as sterile by the manufacturer (Ethox Corporation of Buffalo, NY is a manufacturer of standard blood bags). Cryopharm personnel will record the weight of the "Processed Lyophilization Bag", and load the bag into the sterile lyophilizer chamber when the shelf temperature has equilibrated at  $-20^{\circ}\text{C}$ . The cells will then be frozen in place and lyophilized according to the current standard operating procedures for the lyophilization of human red blood cells. The time and temperature of the drying cycle will depend on the current Cryopharm technology. The current drying procedure will require approximately 170 hours. At the end of the cycle, sterile ultrapure nitrogen will be used to purge the lyophilizer chamber to keep the moisture away from the dried cells before the bag is sealed. The sealed bag will be stored at  $4^{\circ}\text{C}$ , and placed under Q.C. quarantine. Q.C. personnel will inspect the quality of the dried cells, and will determine the percent weight loss of each dry unit. Only the unit that has met all product specifications will be shipped to the study site for reconstitution and transfusion.

## APPENDIX III

### Reconstitution/Wash of Lyophilized Red Blood Cells at Study Site

Cryopharm personnel will be at the study site to supervise preparation of the samples prior to actual transfusion. All buffer solutions for reconstitution will be prepared in the class 100 clean room at Cryopharm. These buffer solutions will be tested for sterility and pyrogenicity by Cryopharm's Quality Control prior to release for clinical use. All buffer components are familiar reagents and most are commercially available as USP grade. The dried sample removed from the refrigerator will be allowed to warm up to room temperature before reconstitution. The reconstitution and cell washing will require a Cobe 2991 cell washer. The Cobe processing set will be installed according to the Operator's Handbook 2991. The dried cells will be reconstituted by adding sterile, reconstitution buffer (37°C). The reconstituted cells will then be washed several times to remove all cryoprotective reagents. It will be washed once with wash buffer for 15 minutes, followed by a 10 minute incubation step and wash with isolation buffer for two minutes, and one more wash with a Buffered Dextrose Saline-based solution for two minutes. The reconstituted cells will then be filtered through a blood filter and placed into an empty, vented red cell labeling vial. The vial will be spun at 1500 xg for 10 minutes. The supernatant and a small aliquot of packed red cells will be removed using a 19 gauge spinal needle for sterility testing and in-vitro analysis. The unit will be accepted for transfusion if the hemoglobin is less than 200 mg/dl, total processing time less than three hours and unit meets specifications. The recovery, indices and osmotic stability assay will be performed on the lyophilized cells.

#### Labeling of Red Blood Cells (Use Aseptic Technique at ALL Times)

1.  $^{51}\text{Chromium}$  (150 to 200 microcuries) are added to the vial containing packed reconstituted red blood cells using a tuberculin syringe which is gently swirled while adding chromium and 30 seconds after the addition.
2. Incubate at room temperature for 30 minutes; swirl the vial 30 rotations by hand every 10 minutes.
3. Add ascorbic acid (100 mg) to the vial.
4. Withdraw 0.5 ml of labeled RBC into a hypodermic syringe and inject into a purple top tube for standard control.
5. Withdraw as much as possible of the packed labeled RBC into a fresh hypodermic syringe.
6. Weigh the filled syringe and empty 18-19 gauge butterfly set.
7. Remove a 5 ml blood sample from patient's catheter into purple-top



8. Attach the butterfly needle on the filled syringe and inject all of the labeled RBC into the patient within 30 minutes of labeling; RECORD TIME! The time of infusion should be less than one minute.
9. Weigh empty syringe and butterfly set after injection of blood.
10. Withdraw blood specimens into purple top tubes from opposite arm at timed intervals (refer to Section 5.4.5).

#### Red Cell Survival By Extrapolated Time Zero Method

1. Inject labeled RBCs into patient at Time Zero.
2. Obtain 5 ml blood samples from contralateral arm at timed intervals per protocol (Section 5.4.5).
3. Two aliquots of 1 ml from each sample are placed in 5 ml test tubes which are then capped, mixed and stored at 4°C until counting in a gamma counter. The samples collected during the first hour post injection will be counted on Day 1, then all samples will be counted after collection of the final sample on Day 5.
4. After collection of the 5, 7.5, 12 and 15 minute and one hour timed blood samples on Day 1, the duplicates of these time points will be counted for 10-30 minutes each in a gamma counter, along with the duplicate background and standard controls. The cpm in these samples will be used to calculate a preliminary extrapolated time zero and cell survival. This preliminary estimate is needed to determine whether the collection of blood samples should continue on the succeeding four days. At this point Dr. Weinstein and Cryopharm's clinical coordinator will determine whether the volunteer needs to return to the hospital for the scheduled blood sampling on days 2, 3, 4, and 5. If further blood collections are not warranted, the volunteer will only return for the scheduled follow-up examination after discharge from the hospital at the end of 24 hours post-injection.
5. Correct the CPM of each timed sample for  $^{51}\text{Cr}$  elution (~1%/day) by multiplying the CPM for each time point by 0.01d where d = day post transfusion. Add these calculated elution values to the observed cpm for their corresponding samples. Correct the cpm of each sample for background by subtracting the mean cpm of the two background samples. Use the elution and background corrected cpm to calculate extrapolated time zero and cell survival.
6. To determine the extrapolated time zero value, convert cpm to net cpm per ml red cells for each sample using the hematocrit. Average the counts of duplicates for each sample. Draw the best fit straight line through data points (5-15 minutes) post-transfusion using logarithmic regression to determine the cpm per ml red cells at time zero by extrapolation to the y axis intercept. The mean corrected cpm of the duplicate 5, 7.5, 12, and 15 minute samples are

plotted versus time on a logarithmic-linear graph. This plot should be used as a check to ensure a reasonable fit.

7. Calculate percent recovery of label for each sample as  $R = (T/S) \times 100$  where  $R = \% \text{ recovery}$ ,  $S = \text{extrapolated Time Zero CPM}$ ,  $T = \text{net mean cpm per ml red cells}$ .
8. Determine apparent  $T_{1/2}$  of  $^{51}\text{Cr}$  survival by plotting  $\% \text{ recovery}$  as a function of time on semi-log paper and drawing the best straight line through the points (normal = 25 to 30 days).
9. If the study is continued through Day 5, all collected samples will be counted for 30 minutes each, and the extrapolated  $t_0$  and cell survival recalculated as above, using all data points.

#### Red Cell Survival by Theoretical Time Zero Method

1. From standard sample, dilute 0.2 ml of labeled red cells with 0.8 ml distilled water in duplicate (total volume = 1 ml).
2. Pipette 1 ml in duplicate of the diluted standard and the blood samples of two 5 ml test tubes.
3. Cap and mix test tubes. Count for 30 minutes in a gamma counter.
4. Correct the cpm for each timed sample for  $^{51}\text{Cr}$  elution ( $\sim 1\%/ \text{day}$ ) by multiplying the cpm for each time point by 0.01 days post-transfusion. Add the calculated elution values to the observed cpm. Correct the cpm of each sample for background by subtracting the mean cpm of the two background samples. Convert cpm to net cpm per ml red cells for each sample using the hematocrit. Average the counts of duplicates for each sample.

5. Total Injected Dose (cpm) =  $(A \times B \times C)$

where:     A = CPM of 1 ml of STANDARD dilutions  
           B = dilution factor of STANDARD  
           C = weight of blood injected into patient (in grams) /  
              1.090 gram per ml

6. Theoretical Time Zero =  $\frac{\text{Total Injected Dose}}{\text{Red Cell Volume}}$

Red Cell volume = (Whole Blood Volume)  $\times$  (Hematocrit on Day 1)  
Where whole blood volume is estimated as shown below.

7. Calculate percent recovery of label for each sample as  $R = (T/S) \times 100$  as before, substituting the theoretical time zero value for  $S$
8. Calculate apparent  $T_{1/2}$  as before using the  $\% \text{ Recovery}$  derived from theoretical time zero.

9. The values for extrapolated  $t_0$  and theoretical  $t_0$  should agree within 10%; otherwise, the  $t_{1/2}$  in the study is suspect because rapid sequestration of labeled red cells can produce a falsely low extrapolated  $t_0$  value (y intercept).

#### Whole Blood Volume

1. Calculate the predicted whole blood volume (WBV) according to the formula:

(for men)  $WBV = 0.3669H^3 + 0.03219W + 0.6041$

(for women)  $WBV = 0.3561H^3 + 0.03308W + 0.1833$

where:  
H = height in meters  
W = weight in kilograms  
WBV = Whole Blood Volume in liters

METABOLIC ACTIVITIES OF FREEZE-DRIED HUMAN ERYTHROCYTES  
(lyophilization/pentose phosphate shunt/methemoglobin/glycolytic  
enzymes)

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Abbreviations: TPI, triose phosphate isomerase; PK, pyruvate  
kinase; PFK, phosphofructokinase; 2,3-DPG, 2,3-  
diphosphoglycerate; ATP, adenosine 5' triphosphate; Hb,  
hemoglobin; metHb, methemoglobin; PPS, pentose phosphate shunt;  
EMP, Embden-Meyerhof Pathway; RBC, red blood cells.

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ABSTRACT: Normal human red blood cells (RBC) were freeze-dried under conditions that caused minimal modification in normal RBC metabolic activities. Because of the known effects of long term storage on metabolic activities and its relationship to posttransfusion survival of stored RBC, we studied the effects of our lyophilization process on RBC metabolism. Metabolic activities of rehydrated lyophilized RBC were compared to non-lyophilized autologous RBC. Of all the metabolic enzymes studied, only triosephosphate isomerase (D-glyceraldehyde-3-phosphate ketol-isomerase, TPI; EC 5.3.1.1 ), enolase (2-phospho-D-glyceratehydro-lyase, En; EC 4.2.1.11) and pyruvate kinase (ATP:pyruvate O<sup>2</sup>-phosphotransferase, PK; EC 2.7.1.40) were decreased when compared to fresh control non-lyophilized RBC. The activities of these enzymes were not significantly different from those of blood bank RBC. Concentrations of high energy intermediates, adenosine triphosphate (ATP), and 2,3-diphosphoglycerate (2,3-DPG), along with lactate and ATP production were decreased in lyophilized RBC. None of the enzymes of the pentose phosphate shunt (PPS) were altered during lyophilization. Since PPS is important in maintenance of the repair and antioxidant defenses of RBC, lyophilized RBC with an intact PPS are thus not so likely to be susceptible to oxidative damage. In addition, our data show that lyophilized RBC possess an intact capacity to: a) synthesize adenine nucleotides; and b) reduce methemoglobin (metHb) to hemoglobin (Hb) and thus maintain the hemoglobin in a functional physiologic state similar to fresh non-lyophilized RBC. The present study thus demonstrates for the first time that the metabolic functions of freeze-dried RBC are maintained upon rehydration and suggest possible use of this lyophilization technology for long term storage of RBC with wide application in transfusion medicine.

The maintenance of the metabolic functions of human red blood cells (RBC) during long term storage is crucial to their in vivo survival and physiologic functions . Several investigators have addressed this problem and have found that certain storage conditions can preserve the metabolic functions of RBC [1-4]. Some of these storage conditions involve refrigeration in liquid media [1,2] which only allows for a 35 to 42 day shelf life or frozen storage at -80 C [3,4]. The latter storage condition requires bulky freezers and maintenance of low temperatures. Neither of these storage conditions allows for convenient handling and transport of human blood cells to the immediate site of traumatic injury, for example, in far-forward combat settings or sites of large scale natural disasters. By contrast, freeze-dried RBC could be stored at ambient temperature and could easily be transported to sites of immediate need. The key question, however, is whether or not lyophilized RBC remain metabolically functional upon rehydration and hence offer the potential of efficacious oxygen delivery upon transfusion . Several studies have shown promise in this regard by demonstrating that certain cryoprotectants are effective in preserving the enzyme activities and functions of dry biological specimens [5].

Normal adult human RBC generate energy almost exclusively through the metabolism of glucose primarily via the Embden-Meyerhof Pathway (EMP) and the pentose phosphate shunt (PPS), Figure 1. These pathways produce the cellular energy crucial to RBC survival and maintenance of proper cell functions [6,7]. In particular, two products of glycolysis, adenosine 5' triphosphate (ATP) and 2,3-diphosphoglycerate (2,3-DPG) act to regulate the oxygen affinity of hemoglobin. In addition, the pathways produce high energy chemical intermediates such as reduced nicotinamide adenine dinucleotide phosphate (NADPH) and nicotinamide adenine dinucleotide (NADH) that serve as important cofactors for glutathione reductase and methemoglobin reductase, respectively. The above glycolytic pathway requires proper

functioning of the various glycolytic enzymes for the formation of the intermediates that are essential if the RBC is to transport oxygen and maintain those physical characteristics required for its in vivo survival in circulation. Abnormal metabolic activities have been observed in RBC that had been stored for extended periods in either conventional liquid media or frozen in glycerol [8-10] and have been implicated in the mechanisms responsible for the rapid removal of transfused RBC.

Since the maintenance of proper metabolic functions of RBC is of such importance in carrying out their physiologic functions, the present study was designed to evaluate the preservation of the key RBC glycolytic enzyme activities following lyophilization and rehydration of RBC. In addition, measurement of the glycolytic enzymes and their intermediates provides an indication of the efficiency of the lyophilization process in preserving the physiologic functions of RBC because of the demonstrated relationship between metabolic activities and in vivo survival of transfused RBC [9,10]. Salient data from the present studies show that metabolic functions of rehydrated lyophilized RBC are comparable to that of blood bank RBC, suggesting the potential application of lyophilized RBC in transfusion medicine.

## MATERIALS AND METHODS

After obtaining informed consent, blood was obtained from six healthy adult individuals with no history of either hemoglobinopathy or abnormal RBC metabolism. Blood was withdrawn from each donor into plastic transfer bags (Fenwal Laboratories, Deerfield, IL) containing 63 mL of citrate phosphate dextrose-adenine (CPD-A) anticoagulant using conventional blood banking techniques. The blood units (500 ml each) were centrifuged at 1800 g for 5 mins at room temperature (22°C) to remove the buffy coat and plasma. The packed RBC were washed in isotonic dextrose saline according to standard washing procedures [11] using an automated cell washer (Model 2991, COBE, Lakewood, CO). The washed and packed RBC (about 85% hematocrit) were resuspended to about 40% in hypertonic phosphate buffered saline containing high molecular weight polymers and cryoprotectants (1800 mOsmol, pH 7.4, Cryopharm Corporation, Pasadena, CA). About 360 g of the RBC suspension were transferred to plastic lyophilization bags (Ethox Corporation, Buffalo, N.Y.). The lyophilization bags were placed in a conventional pharmaceutical shelf freeze-dryer (Virtis Company, Gardiner, N.Y.) and then freeze-dried according to procedures developed by Cryopharm [12]. Samples prepared in this manner consistently exhibit moisture contents of 1-3% as measured by the Karl Fischer method [13]. After 7 days, the dried RBC were rehydrated and reconstituted in phosphate buffered rehydration buffers (360 mOsmol, pH 7.4, Cryopharm Corporation, Pasadena, CA) at 22°C. Briefly, to rehydrate the RBC, 600 g of rehydration buffer were added to the dried RBC and then agitated on a wrist action shaker (Burrell Corporation, Pittsburg, PA) until the RBC were fully rehydrated (usually for 15 mins). At the end of the rehydration step, the RBC suspension was centrifuged at 1800 g for 10 mins at 22°C. The supernatant was removed and the packed RBC were washed twice in special isotonic



wash buffers [12] by centrifugation at 1800 g, using a COBE automatic cell washer. Reconstituted RBC were assayed for glycolytic enzyme activities and intermediates according to previously described methods [14-19]. A control blood sample was obtained from the autologous donor at the time of reconstitution of lyophilized RBC. Control RBC were treated similarly to reconstituted lyophilized RBC with respect to washing.

**Rate of Adenine Nucleotide Synthesis:** The rate of adenine nucleotide synthesis was measured by following the incorporation of  $^{14}\text{C}$ -labeled adenine into the adenine nucleotide pool in intact RBC using the method of Zerez et al [20]. Because all RBC samples had endogenous glucose and exhibited low rates of  $^{14}\text{C}$ -adenine incorporation in the absence of exogenous glucose, a control was included in which incorporation was measured in the presence of 1.0 mM iodoacetate, an inhibitor of glycolysis. This allowed the assessment of "background"  $^{14}\text{C}$ -adenine incorporation (i.e., under conditions of total inhibition of glycolysis) by subtracting  $^{14}\text{C}$ -adenine incorporation in the presence of iodoacetate from incorporation in its absence. Thus, reported rates of  $^{14}\text{C}$ -adenine incorporation were corrected for this "background" incorporation.

**The Rate of Methemoglobin Reduction:** The rate of methHb reduction in intact RBC was determined by using a previously described method [21].

**Other methods:** Rates of ATP and lactate production were determined by the methods described by Beutler [22].

**Statistical Analysis:** Differences between lyophilized and non-lyophilized RBC were analyzed with two tailed Student's t-test for paired data. Comparisons between lyophilized and blood bank stored RBC were made using two tailed Student's t-test for independent data.

## RESULTS

Activities of Glycolytic Enzymes. The results of the measurements of the glycolytic enzyme activities for hemolysates from rehydrated lyophilized RBC and non-lyophilized fresh RBC from autologous donors are summarized in Table 1. The range of enzyme activities for normal RBC and CPD-A stored RBC are included in Table 1 for comparison. The activity of HX in hemolysates was the same in rehydrated lyophilized RBC and non-lyophilized control RBC and was also similar to that for CPD-A stored RBC. The activities of both TPI and PK were reduced in hemolysates from lyophilized RBC, but not different from blood bank stored RBC, Table 1. Hemolysates from lyophilized RBC had TPI activity of 1750  $\mu\text{mol}/\text{min.g}$  Hb which is significantly lower than that of control non-lyophilized RBC, 2140  $\mu\text{mol}/\text{min.g}$  Hb,  $p < 0.005$ . The activity of TPI in blood bank RBC is also higher than that of lyophilized RBC. However, TPI is not a rate-limiting enzyme, since it is present in excess in RBC. The activity of PK in hemolysates of rehydrated lyophilized RBC was 18.9  $\mu\text{mol}/\text{min.g}$  Hb compared to 21.1  $\mu\text{mol}/\text{min.g}$  Hb in non-lyophilized control RBC,  $p < 0.03$ . The activity of PK in blood bank RBC was  $15.0 \pm 2.14$   $\mu\text{mol}/\text{min.g}$  Hb, and this is lower than that of lyophilized RBC. Since PK is a rate-limiting enzyme of glycolysis, the above result implies that enzyme function in lyophilized RBC is equal to or better than enzyme function in blood bank RBC. In contrast, the activities of LDH and DPGM are significantly higher in lyophilized RBC than fresh control non-lyophilized RBC,  $p < 0.001$  and  $p < 0.01$  respectively, Table 1. Note also that lyophilized RBC have PPS enzyme activities that are not significantly different from control non-lyophilized cells. The activities of the remaining EMP enzymes other than the above are similar in

lyophilized, non-lyophilized and blood bank RBC, Table 1.

**Levels of Glycolytic Intermediates.** A comparison of the levels of glycolytic intermediates present in intact rehydrated lyophilized and control non-lyophilized RBC are summarized in Table 2. Significantly higher concentrations of DHAP, 3PG, 2PG, PEP, ADP, and AMP were found in intact lyophilized RBC compared to non-lyophilized control, Table 2. On the contrary, the concentrations of ATP and 2,3-DPG are lower than the values for control RBC. Concentrations of intermediates other than the above were not significantly different from control RBC. Note that the concentrations of intermediates for normal RBC shown in Table 2 are much lower than that for either lyophilized or non-lyophilized RBC. These values were obtained from fresh blood drawn directly into perchloric acid (PCA) and then processed immediately for glycolytic intermediates. In contrast, lyophilized and non-lyophilized control RBC were processed (for example washing of RBC) prior to the isolation of PCA extracts for intermediates and therefore would be expected to be different from normal values.

The rate of lactate production expressed as  $\mu\text{mol/g Hb/hr}$  is significantly reduced in lyophilized RBC,  $6.60 \pm 3.58$  compared to  $10.6 \pm 2.86$  in non-lyophilized control RBC,  $p < 0.001$ ,. Similarly, the rate of production of ATP by lyophilized RBC ( $0.368 \pm 0.173 \mu\text{mol/g Hb/hr}$ ) is also significantly lower than control RBC ( $0.779 \pm 0.305$ ),  $p < 0.01$ ,. The total number of samples evaluated for lactate and ATP productions was 6 for both lyophilized and non-lyophilized RBC.

**Methemoglobin Reduction and Level of Reduced Glutathione.** The semilogarithmic plots of the residual methemoglobin and the incubation time are shown in Fig 2. Methemoglobin reduction in rehydrated lyophilized and non-lyophilized RBC followed similar exponential decay, Fig 2. The rates of methemoglobin reduction,

expressed as the half-life for all the samples tested, are shown in Fig 3. The mean rate of methemoglobin reduction in lyophilized RBC was  $17.9 \pm 4.30$  hr, and not significantly different from control non-lyophilized RBC,  $17.4 \pm 4.80$  hr,  $p > 0.05$ , Fig 3. The concentration of reduced glutathione (GSH) in lyophilized RBC was  $689 \pm 136$   $\mu\text{g/g}$  Hb which is significantly lower than that of non-lyophilized fresh RBC,  $2150 \pm 406$   $\mu\text{g/g}$  Hb,  $p < 0.001$ . Although the concentration of GSH was reduced in lyophilized RBC, preliminary data indicated that the activities of glutathione reductase (NAD(P)H) (NAD(P)H:oxidized-glutathione oxidoreductase, EC 1.6.4.2; lyophilized RBC= 6.86 and non-lyophilized RBC= 6.44  $\mu\text{mol/min.g}$  Hb) and glutathione peroxidase (glutathione:hydrogen-peroxide oxidoreductase, EC 1.11.1.9; lyophilized RBC= 19.9 and non-lyophilized RBC= 21.6  $\mu\text{mol/min.g}$  Hb) were not different from control RBC.

Rate of  $^{14}\text{C}$ -adenine Incorporation. The capacity of lyophilized RBC to synthesize adenine nucleotides (i.e., AMP, ADP and ATP) was measured by following the incorporation of  $^{14}\text{C}$ -adenine into adenine nucleotides. Non-lyophilized RBC had a rate of  $^{14}\text{C}$ -adenine incorporation of  $2.81 \pm 0.40$  nmol/min.ml RBC (mean  $\pm$  1SD,  $n=5$ ). In contrast, lyophilized RBC had a rate of incorporation of  $0.407 \pm 0.122$  nmol/min.ml RBC (mean  $\pm$  1SD,  $n=5$ ) which was significantly decreased ( $p < 0.00005$ ) compared to non-lyophilized RBC. Although lyophilized RBC had a lower rate of  $^{14}\text{C}$ -adenine nucleotide incorporation, these cells' ability to incorporate adenine into nucleotides is noteworthy because only freshly obtained RBC are capable of this function.

Fig 1. The major pathways of glucose metabolism in mature human red blood cells. The arrows represent enzymatic steps.

Abbreviations: NAD, oxidized nicotinamide adenine dinucleotide; NADH, reduced nicotinamide adenine dinucleotide; Pi, inorganic phosphate; ATP, adenosine 5' triphosphate; ADP, adenosine diphosphate; NADP, oxidized nicotinamide adenine dinucleotide phosphate; GSH, reduced glutathione; GSSG, oxidized glutathione; NADPH, reduced nicotinamide adenine dinucleotide phosphate; Hb<sup>++</sup>, reduced hemoglobin; Hb<sup>+++</sup>, oxidized hemoglobin; CO<sub>2</sub>, carbon dioxide. Glucose is degraded to lactate anaerobically via the Embden-Meyerhof pathway on the left or by diversion of glucose-6-phosphate (G-6-P) into the pentose phosphate shunt on the right. Pentose phosphates (R-5-P) generated by this pathway or by nucleoside degradation can be transformed into intermediates of anaerobic glycolysis for further catabolism. NADH serves as cofactor for methemoglobin reductase activity. NADPH is the physiologic cofactor required for maintenance of adequate GSH to combat ambient oxidative stresses.

# EMBDEN-MEYERHOF PATHWAY

## PENTOSE PHOSPHATE SHUNT

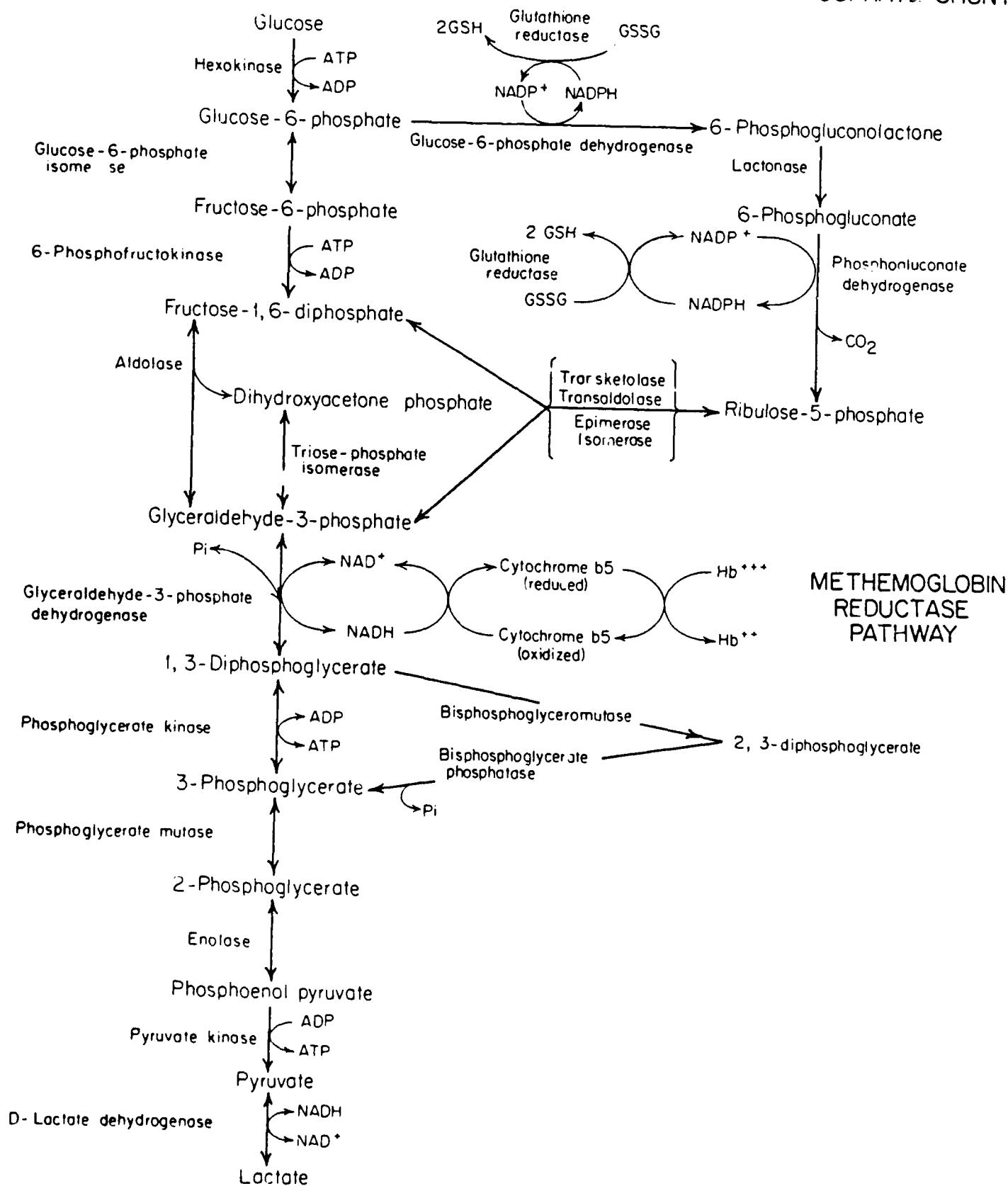


Table 1. Summary of the activities of the glycolytic enzymes in hemolysates from rehydrated lyophilized and non-lyophilized RBC.

Enzyme activity,  $\mu\text{mol/min} \cdot \text{g Hb}$

Enzymes	Lyo	N-lyo	BB	N-R	P
HX	1.26 $\pm$ 0.22	1.65 $\pm$ 0.10	1.20 $\pm$ 0.12	0.98-1.3	NS
PGI	44.7 $\pm$ 4.57	44.3 $\pm$ 2.66	48.3 $\pm$ 6.03	43.7-65.8	NS
PFK	12.1 $\pm$ 1.61	11.7 $\pm$ 0.97	9.73 $\pm$ 2.18	8.44-12.2	NS
Ald	3.59 $\pm$ 0.41	3.72 $\pm$ 0.54	2.39 $\pm$ 0.34	1.97-3.59	NS
TPI	1750 $\pm$ 460	2140 $\pm$ 490	2900 $\pm$ 777	2130-3340	p<0.005
G3PD	318 $\pm$ 68.4	311 $\pm$ 43.0	244 $\pm$ 72.0	238-346	NS
DPGM	5.34 $\pm$ 0.72	4.64 $\pm$ 0.91	8.43 $\pm$ 2.23	3.93-5.90	p<0.015
PGK	340 $\pm$ 147	340 $\pm$ 115	349 $\pm$ 47.7	212-341	NS
PGM	35.2 $\pm$ 5.09	38.1 $\pm$ 5.99	17.3 $\pm$ 6.70	13.9-38.0	NS
Eno	4.99 $\pm$ 0.99	7.60 $\pm$ 0.87	4.96 $\pm$ 0.89	4.2-6.58	p<0.001
PK	18.9 $\pm$ 5.71	21.1 $\pm$ 5.40	15.0 $\pm$ 2.14	12.5-17.2	p<0.032
LDH	231 $\pm$ 29.0	190 $\pm$ 19.2	141 $\pm$ 56.4	145-203	p<0.001
G6PD <sup>s</sup>	12.4 $\pm$ 1.55	14.7 $\pm$ 1.82	ND	9.90-13.2	NS
6PGD <sup>s</sup>	11.1 $\pm$ 0.99	10.0 $\pm$ 1.09	ND	7.27-10.0	NS
TA <sup>s</sup>	0.97 $\pm$ 0.21	1.10 $\pm$ 0.34	ND	0.78-1.32	NS
TK <sup>s</sup>	0.68 $\pm$ 0.13	0.93 $\pm$ 0.66	ND	0.50-1.03	NS

Data represent the mean $\pm$ sd for 6 samples. Data from blood bank stored RBC are included for comparison with rehydrated lyophilized RBC. Blood bank samples (total number=3) were stored at 4°C for at least 10 days before being analyzed. Abbreviations: lyo, lyophilized; N-lyo, non-lyophilized; BB, Blood bank; N-R, normal range; ND, not detected; NS, not significant (comparisons were made between lyophilized and non-lyophilized RBC); HX, hexokinase (ATP:D-hexose-6-phosphotransferase, EC 2.7.1.1); PGI, glucose-6-phosphate isomerase (D-glucose-6-phosphate ketol-isomerase, EC 5.3.1.9); PFK, 6-phosphofructokinase (ATP:D-fructose-6-phosphate 1-phosphotransferase, EC 2.7.1.11); Ald, fructose-bisphosphate aldolase (D-fructose-1,6-bisphosphate D-

glyceraldehyde-3-phosphate-lyase, EC 4.1.2.13); TPI, triosephosphate isomerase (D-glyceraldehyde-3-phosphate ketol-isomerase, EC 5.3.1.1); G3PD, glyceraldehyde-3-phosphate dehydrogenase (D-glyceraldehyde-3-phosphate:NAD<sup>+</sup> oxidoreductase (phosphorylating), EC 1.2.1.12); DPGM, bisphosphoglycerate mutase (3-phospho-D-glycerate 1,2-phosphomutase, EC 5.4.2.4); PGK, phosphoglycerate kinase (ATP:3-phospho-D-glycerate 1-phosphotransferase, EC 2.7.2.3); PGM, phosphoglycerate mutase (D-phosphoglycerate 2,3-phosphomutase, EC 5.4.2.1); Eno, enolase (2-phospho-D-glyceratehydro-lyase, EC 4.2.1.11); PK, pyruvate kinase (ATP:pyruvate O<sup>2</sup>-phosphotransferase, EC 2.7.1.40) LDH, D-lactate dehydrogenase (lactate:NAD<sup>+</sup> oxidoreductase, EC 1.1.1.28); G6PD, glucose-6-phosphate dehydrogenase (D-glucose-6-phosphate:NADP<sup>+</sup>1-oxidoreductase, EC 1.1.1.49); 6PGD, phosphogluconate dehydrogenase (6-phospho-D-gluconate:NAD(P)<sup>+</sup>2-oxidoreductase, EC 1.1.1.43); TA, transaldolase (sedoheptulose-7-phosphate:D-glyceraldehyde-3-phosphate glycerone transferase, EC 2.2.1.2), TK, transketolase (sedoheptulose-7-phosphate:D-glyceraldehyde-3-phosphate glycoaldehyde transferase, EC 2.2.1.1)

§ Enzymes of the Pentose Phosphate Pathway.



Table 2. Comparison of the levels of glycolytic intermediates in rehydrated lyophilized and fresh non-lyophilized RBC

Concentrations of intermediates, nmols/ g Hb

<u>Intermediates</u>	<u>Lyo</u>	<u>N-lyo</u>	<u>NV</u>	<u>P</u>
G6P	49.8±72.1	76.5±102	100±28.0	NS
F6P	0.92±2.26	3.05±7.47	15.6±6.30	NS
FDP	760±425	149±179	4.70±1.60	NS
DHAP	1770±687	174±147	37.5±3.10	p<0.012
GAP	112±46.8	44.9±43.5	9.38±6.30	NS
2,3-DPG	3152±938	9633±2640	13500±2000	p<0.004
3PG	611±210	134±56.1	122±28.0	p<0.006
2PG	338±252	216±165	31.3±13.0	p<0.046
PEP	216±104	67.5±50.8	50.0±16.0	p<0.01
Pyr	170±52.2	193±125	84.4±25.0	NS
Lact	6032±2730	9495±3542	1140±370	NS
ATP	1758±392	3875±780	3220±280	p<0.008
ADP	1743±316	700±133	409±56.0	p<0.003
AMP	2370±343	204±125	134±25.0	p<0.001

Data represent the mean±sd for 6 samples. Normal values are included in the table for comparison with present data.

Abbreviations: lyo, lyophilized; N-lyo, non-lyophilized; NV, normal values; P, probability for comparisons between lyophilized and non-lyophilized RBC.

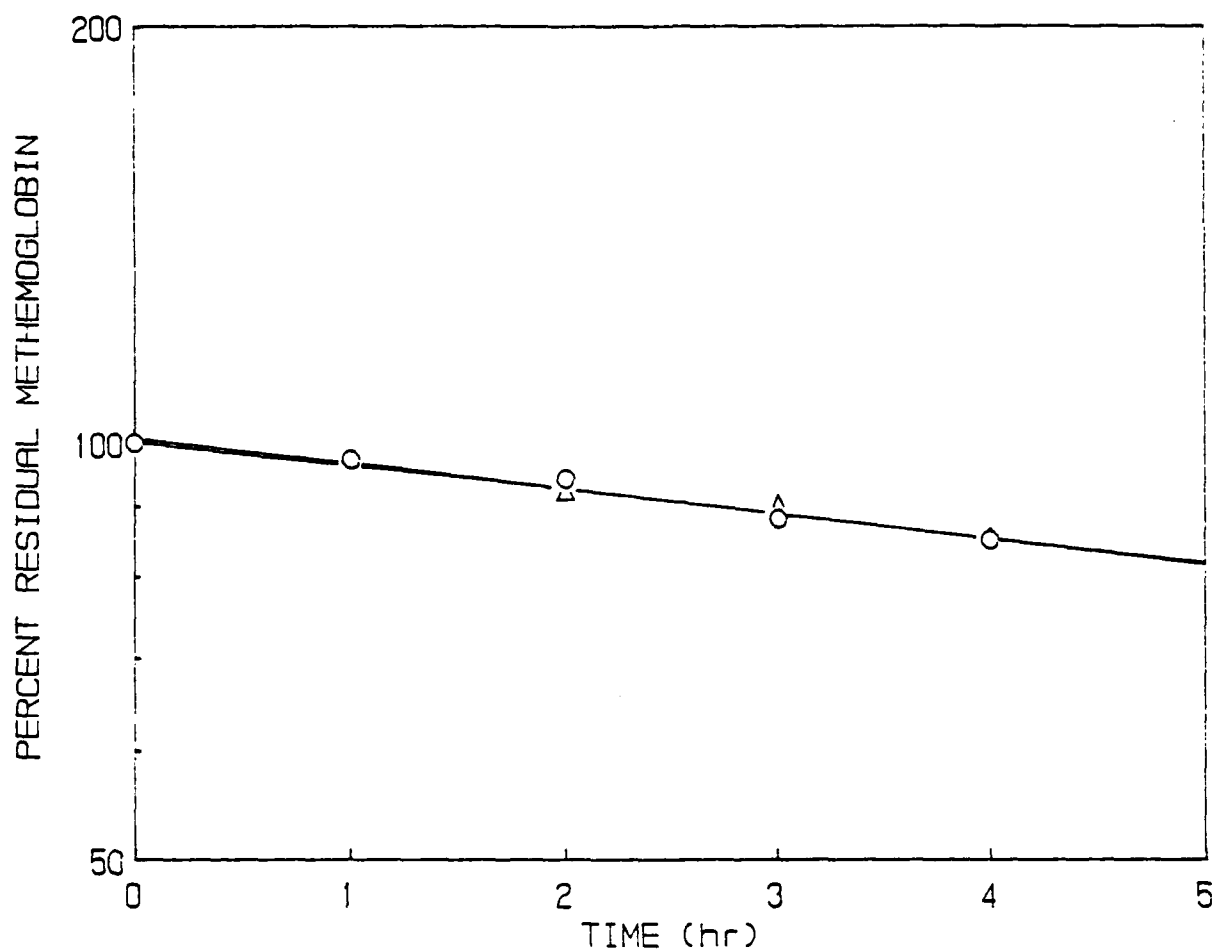


Fig 2. Linear regression fit for semilogarithmic plots of the percent residual methemoglobin versus time of incubation for intact RBC from lyophilized (O) and non lyophilized RBC (Δ). Data are the mean of 6 samples.

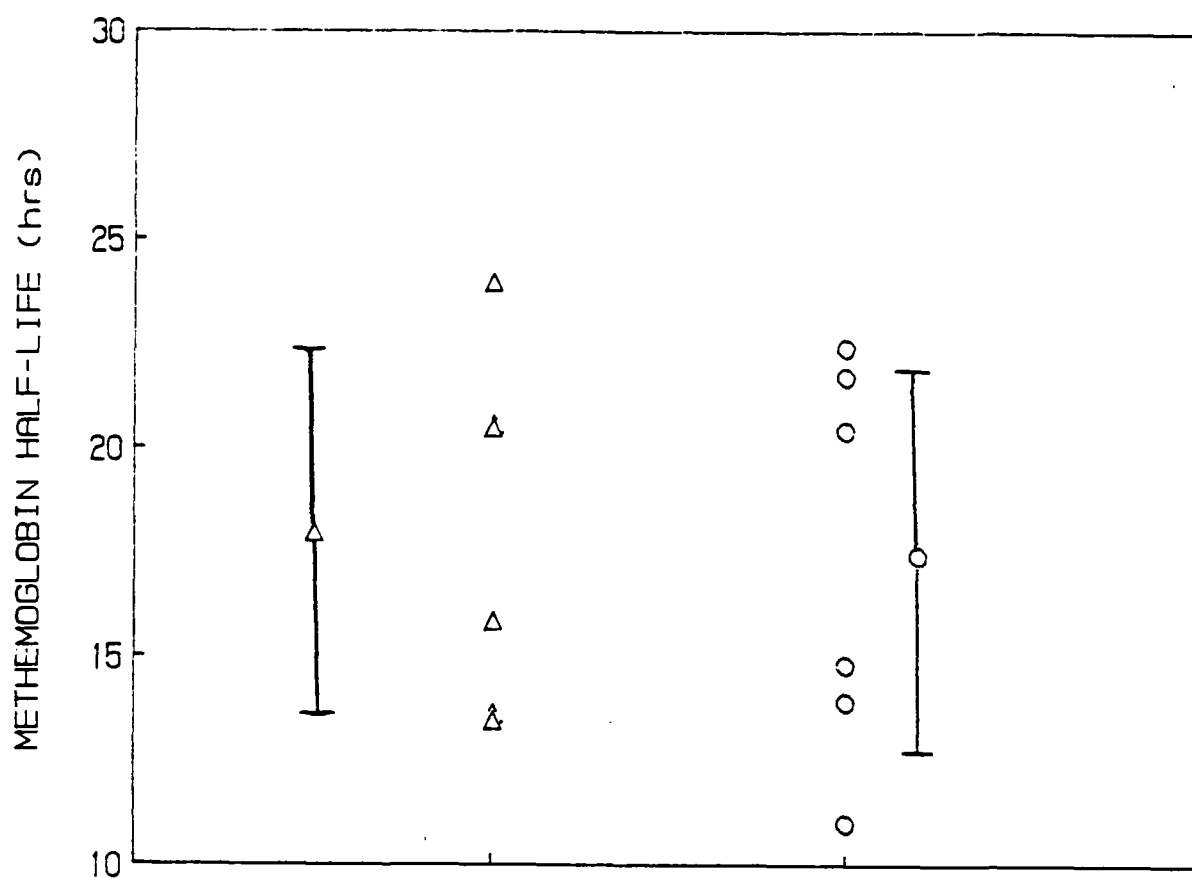


Fig 3. Rate of methemoglobin reduction (expressed as half-life) in intact lyophilized (O) and non-lyophilized RBC (Δ). The mean  $\pm$  s.d metHb half-life for both cell types are also shown.

## DISCUSSION

One of the major problems in blood preservation concerns the metabolic and functional lesions occurring during long term storage of RBC in either conventional liquid media or frozen in glycerol. These storage lesions ultimately result in shortened RBC survival after transfusion [23]. For satisfactory preservation of red blood cells, it is therefore important that the metabolic functions of the cells are well preserved. The relationship between glycolytic enzyme activities and freeze-dry processing of RBC have not been previously studied. We have demonstrated that the activities of the majority of the enzymes involved in RBC metabolism are very well preserved in rehydrated lyophilized RBC. The limitations to overall glycolysis must arise from rate-limiting enzyme activity in the sequence of glycolysis. It is known that among the glycolytic enzymes tested, HX has the lowest activity [24]. The first step in phosphorylation, therefore, may be the rate-limiting step for overall glycolysis. Also, measurement of the free energy changes at various steps of glycolysis identified PFK and PK as potential controlling steps for various portions of the glycolytic pathway [25]. Our data showed that of all the enzymes studied, only TPI, ENO and PK are significantly decreased in lyophilized RBC when compared to fresh control non-lyophilized RBC. However, the activities of these enzymes are not significantly different from that obtained with blood bank-stored RBC. The activity of TPI is the highest of any of the glycolytic enzymes by one or two orders of magnitude and may not play an important role in controlling glycolysis [24]. The fact that all the major rate-limiting enzyme activities are not different from that of fresh samples strongly suggests that the glycolytic pathway is functional in lyophilized RBC. It is interesting to also note that the activities of both PFK and G6PD have been found to be reduced in RBC stored in conventional liquid media for an extended period [26]. The impact of such changes in activities of PFK and G6PD on the glycolytic pathway and post-transfusion survival remains to be determined. The

activities of these enzymes are within the normal range in lyophilized RBC. In addition, none of the PPS enzymes were decreased by the lyophilization process. Since the major function of PPS is to produce NADPH and GSH which together provide the main line of defense for RBC against oxidative injury, lyophilized RBC with intact PPS are not as likely to be susceptible to oxidant damage.

The methemoglobin reduction pathway is another important component of RBC metabolism. Decreased activity of this pathway may lead to accumulation of methemoglobin and a loss of oxygen transport capabilities of RBC in as much as the metheme portion cannot combine with oxygen. Our results show that lyophilized cells have intact capacity to reduce methemoglobin to functional hemoglobin. The maintenance of both methemoglobin reduction and intact PPS suggest that lyophilized cells are not as likely to undergo hemoglobin oxidation. Decreased antioxidant defenses in CPD-A stored blood has been implicated as the mechanism responsible for the rapid removal of transfused RBC [26]. The fact that these defenses are still maintained in rehydrated lyophilized RBC further demonstrates the efficiency of lyophilization technology in preserving the vital functions of RBC. Note also that PPS is important in the production of 5-phosphoribosylpyrophosphate (PRPP) which is used by the RBC for the synthesis of adenine nucleotides. These data also show that lyophilized RBC with their intact PPS are able to utilize an exogenous adenine source in the synthesis of adenine nucleotides .

Although the concentrations of certain key glycolytic intermediates (ATP, 2,3-DPG) are decreased in lyophilized cells, sufficient quantities of these intermediates remain for viability and continuation of the various ATP and 2,3-DPG dependent functions as they were prior to lyophilization. In any event, the concentrations of ATP and 2,3-DPG in lyophilized cells are similar to that of RBC in blood banking in the course of their current storage life.

The above data thus show that rehydrated, lyophilized RBC are

at least comparable metabolically to blood bank RBC and not too different from fresh non-lyophilized RBC. The advantage of lyophilized RBC rests in the ability to store them for longer periods under storage conditions that are far easier to achieve (i.e., room temperature), making transport to sites of immediate need a more feasible task.

In conclusion, our data show for the first time that freeze-dried RBC maintain their metabolic functions upon rehydration. Since RBC viability is related to the physiologic functions of the cells, the lack of proper metabolic function should not be a factor for the ability of lyophilized RBC to provide efficacious oxygen delivery upon transfusion comparable to that of blood bank RBC. These results thus offer the exciting prospect of a wide application of freeze-dried RBC in transfusion medicine and thus warrant further investigation of these cells.

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1. Strumia, M. M., Eusebi, A. J. & Strumia, P. V. (1967) J. Lab. Clin. Med. 71, 138-147.
2. Beutler, E. (1974) in The Human Red Cell in Vitro, eds. Greenwalt, T. J. & Graham, A. J. (Grune & Stratton, New York), pp. 189-216.
3. Derrick, J. B., Lind, M. & Rowe, A. W. (1969) Transfusion 9, 317-323.
4. Valeri, C. R. (1974) in The Red Blood Cell, ed. Surgenor, D. M. (Academic Press, New York), Vol. 1, pp. 511-574.
5. Crowe, J. H., Crowe, L. M. & Jackson, S. A. (1983) Arch. Biochem. Biophys. 220, 477-484.
6. Brewer, G. J. (1974) in The Red Blood Cell, ed. Surgenor, D. M. (Academic Press, New York), Vol. 1, pp. 473-508.

7. Harmening-Pittiglio, D. (1989) in Modern Blood Banking and Transfusion Practices, ed. Harmening-Pittiglio, D. (F.A. Davis Company, Philadelphia), 2nd Ed., pp. 1-23.
8. Dern, R. J., Brewer, G. J. & Wiorkowski, J. J. (1967) J. Lab. Clin. Med. 69, 968-979.
9. Nakao, K., Wada, T., Kamiyama, T., Nakao, M. & Nagano, K. (1962) Nature, 194, 877-878.
10. Jones, N.C. H., Mollison, P. L. & Robinson, M. A. (1957) Proc. Roy. Soc. (London) 147, 476-497.
11. COBE (1985) in Blood Cell Processor (COBE Laboratories Inc. Colarado) pp.1-3.
12. Goodrich, R. P. (1989) USA Patent Serial Number 4,874,690.
13. May, J. C., Grim, E., Wheeler, R. M. & West, J. (1982) J. Biol. Stand. 10, 249-259.
14. Beutler, E. (1984) in Red Cell Metabolism: A Manual of Biochemical Methods. (Grune & Stratton, Orlando) 3rd., Ed., pp.38-126.
15. Minakami, S., Suzuki, C., Saito, T. & Yoshikawa, H. (1965) J Biochem. 58, 543-550.
16. Lowry, O. H., Passonneau, J. V., Hasselberger, F. X. & Schulz, D. W. (1964) J. Biol. Chem. 239, 18-30.
17. Tanaka, K. R. (1969) in Biochemical Methods in Red Cell Genetics, ed. Yunis, J. J. (Academic, San Diego) pp. 167-181
18. Matschinsky, F. M., Kaufman, F. C. & Ellerman, J. E. (1968) Diabetes 17,475-480.
19. Koutras, G. A., Hattori, M., Schneider, A. S., Ebaugh, F. & Valentine, W. N. (1964) J. Clin. Invest. 43,323-331.
20. Zerez, C. R., Lachant, N. A. & Tanaka, K. R. (1989) J. Lab. Clin. Med. 114,43-50.
21. Zerez, C. R., Lachant, N. A. & Tanaka, K. R. (1990) Blood 76, 1008-1014.
22. Beutler, E. (1984) in Red Cell Metabolism: A Manual of Biochemical Methods. (Grune & Stratton, Orlando) 3rd., Ed., pp.122-124, 144-145.
23. Wolfe, L. C. (1985) Transfusion 25, 185-203.

24. Brewer, G. J. (1974) in The Red Blood Cell, ed. Surgenor, D. M. (Academic Press, New York), Vol.1, pp.387-433.
25. Yoshikawa, H. & Minakami, S. (1968) Folia. Haematol.89,357-375.
26. Noble, N. A., Tanaka, K. R. & Johnson, D.E.(1982) Am. J. Hematol 13, 1-8.