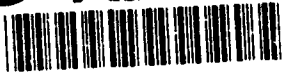


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Research Progress Report
submitted to the
U.S. Naval Medical R & D Command

Freeze-Dried Human Red Blood Cells
Contract No. N00014-90-C-0053

April 15, 1992

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CRYOPHARM

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RESEARCH PROGRESS REPORT
SUBMITTED TO THE US NAVAL MEDICAL RESEARCH AND DEVELOPMENT COMMAND
FREEZE-DRIED HUMAN RED BLOOD CELL
CONTRACT NO. N00090-C-0053

CRYOPHARM CORPORATION

April 15, 1992

Statement A per telecon Chris Eisemann
NMRDC/04A
Bethesda, MD 20889-5044

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CRYOPHARM

April 14, 1992

Defense Technical Information,
Building 5,
Cameron Station,
Alexandria, VA22304-6145.

Dear Sir/Madam:

Enclosed is Cryopharm's seventh research progress report on lyophilized human red blood cells (Contract No. N00014-90-C-0053). This research report focuses on Cryopharm's third clinical study, and summarizes our progress on lyophilized human red blood cells (RBC). We present results of Cryopharm's initial in vivo survival study and demonstrates that by using the current Cryopharm's lyophilization procedure, human red blood cells can be successfully lyophilized, reconstituted and infused into a healthy donor with 86% post-transfusion survival. This level of in vivo survival of lyophilized RBC is comparable to fresh non-lyophilized human red blood cells. We also present results that show that reconstituted, lyophilized human red blood cells can be stored in standard blood bank preservatives with excellent maintenance of glycolytic intermediates comparable to that of refrigerated blood.

Please feel free to call me if you would like to discuss our results and future plans.

Sincerely,


Samuel O. Coker, Ph.D.,
Project Manager Red Cell Research.

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Section 2

Protocol for estimating the in vivo survival of lyophilized RBC in cynomolgus monkeys

Section 3

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SUMMARY

This research report focuses on Cryopharm's third clinical study, and summarizes our progress on lyophilized human red blood cells (RBC) since our last progress report submitted to the Naval Medical Research and Development Command on January 15, 1992. The present clinical study was conducted in both human volunteers and in two Cynomolgus monkeys.

Animal Study: In this study low doses (3-6ml) of packed reconstituted, lyophilized RBC were labelled with radioactive sodium chromate (^{51}Cr) and then infused into two healthy cynomolgus monkeys. The rehydrated cells were washed twice with dextrose saline prior to labelling with chromium. The data from this initial study show that reconstituted, lyophilized monkey RBC have 24 hour in vivo survival of 94 and 87 % in monkey # 344 and #316 respectively. Fresh non-lyophilized RBC from cynomolgus monkey have normal in vivo survival of 88%. This level of in vivo survival of reconstituted, lyophilized RBC is comparable to that of fresh non-lyophilized RBC.

In addition to determining the 24 hour in vivo survival of transfused RBC in monkeys, blood samples were collected from each monkey for 6 days post -infusion to measure the half life of the circulating RBC. The results from this extended study show that reconstituted, lyophilized RBC have a half life of between 8 and 10 days which is comparable that of fresh non-lyophilized RBC (about 6-8 days), Figures 2A, 2B .

In the two animals studied there was no indication of any side effect or discomfort as a result of the transfused quantity of lyophilized RBC. This animal study was conducted at TSI Mason Research Institute at Worcester, MA. The present survival study thus demonstrate that red blood cells from cynomolgus monkeys can be lyophilized according to Cryopharm's procedure such that normal in vivo survival can be obtained upon reconstitution and transfusion into an autologous donor. In addition, the results from our animal survival study indicate that primates can be used as models to further evaluate the in vivo behavior of our lyophilized RBC.

Human Study: Following a successful demonstration of significant level of cell survival in primates, Cryopharm has initiated red cell survival studies in healthy human subjects. This clinical study was designed according to established standard procedures for red cell sequestration studies to evaluate splenic function, in vivo survival of RBC and also to diagnose hemolytic anemias. The clinical study is being conducted at St. Elizabeth's Hospital Blood Transfusion Center in Boston, MA, with informed consent of the volunteer and hospital Institutional Review Board approval. In this study, 14 ml of packed chromium labelled reconstituted, lyophilized human red blood cells were infused into a healthy

autologous donor. Peripheral blood samples were collected at different time points for up to five days post-infusion to measure the levels of circulating ^{51}Cr -labelled RBC. Urine samples were also collected from the volunteers during the first 24 hours post-infusion for estimation of the level of chromium clearance by the kidney. The volunteer was subjected to gamma camera imaging and external probe counts over the heart, spleen, and liver at 4 hours and 24 hours post-infusion, to determine the organ distribution of the injected chromium-labelled reconstituted, lyophilized RBC. A total of 3 volunteers will be used for this initial evaluation of the in vivo survival study of reconstituted, lyophilized RBC. Results from the first volunteer show that :

1. Chromium labelled reconstituted, lyophilized human RBC have in vivo survival of about 86%. This level of extended circulation is comparable to that which has been reported for normal fresh non-lyophilized red blood cells. It is generally accepted that once the RBC have survived the initial 24 hours post transfusion period, the remaining RBC are capable of a normal in vivo life span of about 120 days period. According to regulatory standards of the American Association of Blood Banks, an in vivo survival of at least 70% of the transfused cells 24 hours after transfusion is sufficient to qualify a storage method as acceptable.
2. Gamma imaging of the three highly vascularized organs of the body (heart, liver and spleen) show a uniform distribution of chromium labelled reconstituted, lyophilized RBC. These distribution profiles are similar to that for normal fresh RBC. This data indicates that there was no sequestration of the lyophilized RBC into any particular organ.
3. External probe counts taken over the heart, liver and spleen to determine the distribution of the lyophilized RBC at 4 hours and 24 hours post transfusion showed approximately equal proportions over each organ. These results thus agree with that obtained with the gamma imaging technique and are indicative of a normal cell distribution profile.
4. Urine excretion of the isotopic label is about 4% of the injected radioactivity. This level of loss of isotope in the urine is relatively low and well within the acceptable limit for fresh non-lyophilized RBC. This reflects normal elution of chromium by the body.
5. There were no significant changes in the vital signs (body temperature, blood pressure, and heart rate) of the volunteer during the study.

The above in vivo survival data thus demonstrates that by using the current Cryopharm's lyophilization procedure, human red blood cells can be successfully lyophilized, reconstituted and infused into a

donor with retention of normal *in vivo* survival pattern as measured by a single isotope technique. There was no differential sequestration of the reconstituted, lyophilized RBC into highly vascularized regions of the body such as the heart, spleen and liver. Reconstituted cells maintained normal cell indices and rheological parameters which are essential if the reconstituted lyophilized RBC are to carry out their normal physiologic function. Excretion of chromium into the urine appears to be within the normal range for an *in vivo* survival study using a single isotope method. In addition, since satisfactory *in vivo* survival is one of the main considerations in assessing the quality of transfused RBC, our *in vivo* survival data demonstrates a successful step in the direction of a lyophilized red cell product for transfusion medicine.

Our basic research efforts since our last Progress Report of January 15, 1992, have been directed at : (1) development of buffer formulations based on the glass transition and water replacement theory; (2) establishing standard lyophilization procedures such that normal cell indices are maintained upon rehydration; (3) evaluating the stability of our lyophilized RBC at different storage conditions. Results of our clinical *in vivo* survival data show that research objectives 1 and 2 stated above, have been met (i.e., development of a buffer formulation that will adequately protect the RBC during lyophilization; and secondly development of a successful lyophilization cycle). Preliminary results from our storage stability studies show that:

1. Upon rehydration, red blood cells that had been stored in lyophilized state for at least 7 days maintained cell properties that were either equal to or better than control fresh non-lyophilized RBC that had been stored for comparable period in the liquid state.
2. The levels of glycolytic intermediates (ATP, adenosine 5'triphosphate; 2,3-DPG 2, 3-diphosphoglycerate) in rehydrated, lyophilized RBC were much higher than that for control non-lyophilized RBC. In addition , the level of lactate was significantly increased in control samples, $p > 0.001$. These results taken together suggest that metabolic activities were effectively arrested during storage in the lyophilized state while control fresh RBC continued to metabolize glucose normally.
3. Rehydrated, lyophilized RBC that were stored in standard blood bank preservatives (CPDA, ADSOL and AS) demonstrated excellent maintenance of ATP and 2,3-DPG well above that for control RBC that were stored in CPDA for a comparable period in the liquid state. These results indicate that normal cell properties were preserved during our lyophilization procedures, and that the cells can continue to metabolize normally after reconstitution.

In addition to the above studies, our basic research activities have also been directed at developing rehydration procedures that will eliminate the need for washing the cells. Results obtained so far indicate that our lyophilized RBC can be rehydrated with a two-step procedure. Buffers are now being evaluated with respect to their effectiveness in rehydrating lyophilized RBC such that any additional wash will be unnecessary (a one-step ,no wash reconstitution process).

In the process development area , our research has focussed on developing the optimum lyophilization conditions. The lyophilization process has been improved such that multiple samples can now be lyophilized with a standard deviation of less than 10% in apparent dryness between the various samples. We are also currently evaluating different sample configurations and container designs in order to improve the efficiency of our lyophilization procedures.

UPDATE OF PROJECT STATUS RELATIVE TO 1989 MILESTONES

Cryopharm submitted its original research proposal on lyophilized red blood cells in September 1989. In that proposal we included a chart of research milestones, a copy of which is also included in this report for reference, Table 1. In our progress reports of November 1991 and January 1992, we outlined all our progress to date with respect to the proposed projects and we showed that most of the proposed studies had been completed at the specified projected dates. The results from our present *in vivo* survival studies of reconstituted, lyophilized autologous red blood cells in both human volunteer and Cynomolgus monkeys are included in this report. The reconstitution and washing procedures that are now being used in the present clinical study have been extensively modified such that a total of 10-15 minutes is required . The quality of the cells that are obtained at the end of reconstitution is such that we it will be possible to develop a "NO WASH" procedure. Taken together, all the results detailed in this report show that all the research objectives outlined in our 1989 milestone chart have been met.

Our final proposed 1989 milestone was the filing of an IND for phase I clinical trials at the end of Year 3 (May 1993). With our present successful preliminary clinical study, we anticipate filing for an IND by the end of this year, ahead of schedule.

RESEARCH PROGRESS REPORT

Background

In the January 1992 progress report, we outlined Cryopharm's basic red cell research development of preparative procedures that allowed us to freeze and dry human red blood cells such that at least 80-90% of normal cell indices (mean cell volume, MCV; mean cell hemoglobin, MCH; and mean cell hemoglobin concentration, MCHC) were obtained upon rehydration. We showed that our reconstituted, lyophilized RBC have normal rheological properties. Normal rheological properties are required if the RBC are to carry out normal physiologic function of efficient oxygen transportation to the tissues. With our new buffer formulations and processing conditions we have been able to obtain cells which are far superior with respect to overall quality than those used in previous clinical trials.

Given these improvements in the *in vitro* parameters, Cryopharm's objective for the third clinical study has been to determine if the observed improvements in the *in vitro* parameters of osmotic stability, osmotic deformability, filterability and hematological indices (MCV, MCH, and MCHC) for reconstituted, lyophilized cells lead to significant increase in *in vivo* survival.

IN VIVO SURVIVAL STUDY

A. Cynomolgus Monkey: The choice of the cynomolgus *macaca fascicularis* monkey as a model for investigating the *in vivo* survival of reconstituted, lyophilized red blood cells was made after *in vitro* studies indicated that red blood cells from rats were not capable of surviving the initial lyophilization procedures developed for human red blood cells. Preliminary studies with cynomolgus monkeys showed that their red blood cells can be freeze-dried and reconstituted with the same procedures developed for human red blood cells. The use of an animal model is predicated by the belief that a successful model can be used to optimize our lyophilization procedures. A copy of the protocol for the determination of the *in vivo* survival of reconstituted, lyophilized RBC is included in this report (Section 2). Briefly, red blood cells from cynomolgus monkeys were lyophilized in buffer developed by Cryopharm. The lyophilized monkeys red blood cells (mRBC) were reconstituted, and washed in standard blood bank dextrose saline. The washed mRBC were labelled with radioactive sodium chromate according to established procedures for estimating *in vivo* survival of red blood cells in the circulation. Two monkeys were used for this study. Each monkey received a single administration of the autologous, lyophilized and reconstituted, chromium labelled mRBC via an intravenous catheter in the peripheral saphenous vein. Following administration of labelled mRBC into each monkey, 0.2ml of blood was collected into

heparinized tubes from a vessel other than the one used for administration of labelled RBC. Blood samples were collected at the various timepoints shown in Table 3A-3B. Circulating half life of the transfused RBC was calculated using a previously described method [1] Preliminary results from the present study showed that:

- * Upon rehydration, lyophilized red blood cells from cynomolgus monkeys maintained normal *in vitro* parameters, Table 2.
- * Reconstituted, lyophilized monkey red blood cells showed 87-94% *in vivo* survival after 24 hours, Tables 3A-3B, and Figure 1. Note that fresh non-lyophilized red blood cells from monkey # C793 showed *in vivo* survival of about 88% after 24 hours. The level of red blood cells circulating *in vivo* after the lyophilization procedures are comparable to that obtained with fresh non-lyophilized red blood cells.
- * Circulating half life of chromium labelled reconstituted, lyophilized RBC from cynomolgus monkeys is about 8-10 days, Figures 2A-2B. This level of extended *in vivo* survival is comparable to that of fresh non-lyophilized RBC from cynomolgus monkey which has been previously estimated to be about 6-8 days.

In overview the present data showed very clearly that lyophilized, reconstituted mRBC have *in vivo* survival that is comparable to that of fresh non lyophilized red blood cells.

B. Human Clinical Study

Following a successful demonstration of cell survival in primates, Cryopharm has initiated red cell survival studies in healthy human subjects. This study was designed to determine if human red blood cells could be lyophilized, reconstituted and then infused into a healthy autologous volunteer without any alteration in *in vivo* survival. An updated clinical protocol is provided in Section 3 of this report. This protocol describes in detail the study procedures and the methods used for isotopic labelling of reconstituted cells with radioactive sodium chromate (^{51}Cr). Briefly, after admission into the study protocol, the volunteer was phlebotomized and 450ml of blood were collected in standard blood bank citrate-phosphate-adenine anticoagulant. Packed red blood cells were isolated from whole blood according to standard blood bank procedures and then lyophilized according to procedures developed by Cryopharm. The lyophilized RBC were sent to St. Elizabeth's Hospital Blood Transfusion Center, Boston for reconstitution and subsequent infusion into the autologous donor. At St Elizabeth's Hospital, the lyophilized RBC were rehydrated according to the protocols established at Cryopharm. The

reconstituted, lyophilized RBC were washed twice in dextrose saline according to standard blood bank practices. The washed RBC were labelled with radioactive sodium chromate (^{51}Cr) according to standard hematologic procedures for estimating in vivo survival of transfused red blood cells. The labelled RBC were infused through a 20 gauge needle into the volunteer via a scalp vein in the right arm. Following the administration of labelled RBC, peripheral blood samples were collected at different time points from an indwelling catheter with a heparin lock inserted into a large vein in the subject's left forearm. Urine collections during the first 24 hours post-infusion were also counted to measure the level of chromium clearance via the kidneys. In addition, gamma camera imaging and external probe counts over the heart, spleen and liver at 4 and 24 hours post-infusion were performed to determine the organ distribution profile of the injected RBC. Note that the design of this clinical study followed the standard procedures for red cell sequestration studies to evaluate splenic function, diagnose hemolytic anemias, or estimate the in vivo survival of RBC in circulation.

Experimental Results from Human Clinical Study

A. **In vitro Parameters:** Prior to infusion of reconstituted, lyophilized RBC into the volunteer, standard in vitro parameters were measured immediately after rehydration and washing to determine if these values are within the expected normal ranges and are shown in Table 4. In vitro parameters from normal fresh non-lyophilized RBC are included for comparison with the lyophilized sample. Note that all the measured in vitro parameters of the reconstituted, lyophilized RBC are all within the expected normal values.

B. **Determination of 24 hour in vivo survival:** Following isotopic labeling each volunteer's own cells were infused according to standard procedures outlined in the clinical protocol. In Table 5 we show the ^{51}Cr radioactivity levels in the peripheral blood samples collected at different time points. The raw data are also expressed as a percent of the injected dose corrected for dilution in the whole blood volume as estimated from body weight and height (reference clinical protocol, section 3). These values agree very well with the extrapolated time-zero which is indicative of normal in vivo stability of transfused, lyophilized RBC.

Chromium labelled reconstituted, lyophilized RBC have a 24 hour in vivo survival of about 86%, Table 5 and Figure 3. This level of extended circulation in vivo is comparable to that which has been reported for normal fresh non-lyophilized red blood cells. It is generally accepted that once the RBC have survived the initial 24 hour post transfusion period, the remaining RBC are capable of a normal in vivo life span. In order to calculate half life of the transfused, lyophilized RBC, peripheral blood samples are being collected over a 21 day period. According to regulatory standards of the

American Association of Blood Banks, an *in vivo* survival of at least 70% of the transfused cells 24 hours after transfusion is sufficient to qualify a storage method as acceptable.

C. Gamma Imaging and Uptake Probe- Distribution of Transfused RBC into Different Organs

In figure 4, we show the distribution of the radio labelled reconstituted, lyophilized RBC through the three highly vascularized organs of the body (heart, liver and spleen) measured using a gamma imaging technique. The high density of radioactivity in the spleen, heart, and the liver indicates that the transfused cells are approximately equally distributed in these organs. The raw counts from the gamma imaging are still being evaluated and will be available in our next report. In Table 6, the raw data shows the proportions of the total radioactivity counts found over the heart, liver and spleen as measured by the external uptake probe. The uptake data are also expressed as dose ratios, Table 6. Note that for standard red cell sequestration studies using fresh chromium labelled RBC from healthy volunteers, the normal distribution profile is about the same in the three organs; (i.e., 1:1:1 , Heart: Liver: Spleen) ratio of distribution. The proportions of the chromium labelled , reconstituted, lyophilized RBC in the heart, spleen and liver as measured with an external uptake probe is similar to that of normal non-lyophilized RBC. The results obtained with the external probe thus correlate with the results obtained with the gamma imaging technique and are indicative of a normal cell distribution profile.

D. Measurement of the Levels of Radioactive Chromium Excreted in Urine.

Urine samples were collected at different time points to determine the level of chromium cleared by the kidney. In any red cell *in vivo* survival study using radioactive chromium, elution of free ^{51}Cr from hemoglobin occurs at a reproducible rate of about 1% per day post transfusion (reference). Free ^{51}Cr is rapidly filtered by the kidneys and excreted in the urine . In addition, release of free ^{51}Cr -tagged hemoglobin by intravascular lysis can also lead to hemoglobinuria if the amount of hemoglobin saturates the level of serum haptoglobin. Urine excretion of the isotopic label is about 4.4% of the injected dose, Table 7. This level of loss of isotope in urine is relatively low and well within the acceptable limit for fresh non lyophilized RBC. This reflects normal elution of chromium by the body.

E. Measurement of Vital Signs of Volunteer. No changes in vital signs (body temperature, blood pressure, heart rate etc.,) occurred after infusion of autologous reconstituted, lyophilized RBC , Table 8. No associated side effects were observed during the study.

Conclusions

This preliminary set of data demonstrates that, using the present Cryopharm lyophilization procedure, human RBC can be successfully lyophilized, reconstituted and infused into a donor with retention of normal in vivo survival. In addition, the data show that there was no differential sequestration of the reconstituted lyophilized RBC into highly vascularized organs of the body by external monitoring of the heart, liver and spleen. The rate of clearance of released chromium into urine via the kidney is within the acceptable normal range. No changes in the vital signs of the subject were associated with the low starting volume of transfused, reconstituted, lyophilized RBC. The above results along with our in vivo survival data from cynomolgus monkeys show that reconstituted, lyophilized RBC are capable of extended in vivo survival that is well above the level recommended for qualifying a suitable storage method by the American Association of Blood Banks.

2. Basic Red Cell Research

In our progress report of January 15, 1992, we recognized the need to determine the stability of our lyophilized RBC at various temperatures. The stability of lyophilized preparations during prolonged storage can be predicted quantitatively from the results of short-term degradation studies at elevated temperatures. This procedure is based on the observation that some measurable quality of the cells such as the deformability and filterability will decline linearly with time at the selected temperature. We have used Arrhenius kinetics to determine the shelf life of two of our buffer formulations one of which is being used for lyophilization of RBC for the ongoing in vivo survival study. The principles in Arrhenius kinetics is predicated by the belief that the reaction rate at a given temperature is dependent on the activation energy of a molecule and the frequency of molecular collisions. As the temperature of a product increases, the activation energy increases. The rate of degradation of the lyophilized product can therefore be determined from the Arrhenius relationship for thermal degradation.

$$\text{Log } K = - (H_a/2.303R) (1/T)$$

In the above equation, K is the specific rate of degradation of the lyophilized product, R is the gas constant and H_a is the heat of activation. Using the Arrhenius equation the shelf life of the lyophilized product can be predicted with a high degree of confidence. Experiments are now ongoing to determine the storage stability of our lyophilized RBC at different storage temperatures (from -25C to + 22C). Results from this work will be presented in the next progress report.

In addition to determining the shelf life of our lyophilized RBC experiments have also been conducted to determine the stability of reconstituted, lyophilized RBC. The main purpose of this study

was to determine whether human red blood cells can be lyophilized such that normal metabolic, rheological and cellular properties are maintained upon rehydration and after extended storage in standard blood bank preservative solutions.

A. Refrigerated Storage of Lyophilized and Rehydrated, Lyophilized Human Red Blood Cells.

The maintenance of normal metabolic, biophysical and biochemical properties of human red blood cells (RBC) during long term storage is crucial to their *in vivo* survival and physiologic functions [2-5]. It has long been believed that the progressive decrease in the viability and *in vivo* survival observed for blood bank stored RBC is due in large part to significant alteration in both the metabolic status and rheological properties of the cells [3,4]. For example, red cell survival can be improved after 8 weeks storage by rejuvenation of ATP levels with adenosine [6]. These rejuvenation procedures are accompanied by an improvement or normalization of membrane-related functions such as morphology, filterability, deformability, osmotic stability and whole blood viscosity [5]. Because of the above reasons [2,3], several studies have been undertaken in order to develop procedures that would prevent the loss of ATP, 2,3-DPG and viability of RBC stored for extended periods. Current storage of human red blood cells involve refrigerated storage of RBC in citrate-phosphate preservative solutions which allow for a 35-45 day shelf life. Frozen storage at -80C permits longer term shelf life (up to 10 years) but requires maintenance of low temperature. Frozen storage also requires the need for low temperature freezers and the requirement to extensively wash the thawed cells to remove the glycerol. This latter restriction precludes the effective utilization of deglycerolized RBC.

Lyophilized storage of red blood cells may provide a useful storage alternative. Removal of over 70% of the available water in packed RBC creates a RBC composition than can tolerate storage temperatures that are far easier to achieve. Both the shelf life and transportation logistics can be improved over existing refrigerated or frozen storage. In order for human red blood cells to fulfill their normal oxygen-carrying functions, proper cell metabolism and membrane properties must be preserved. The present study was therefore designed to critically evaluate the effects of lyophilization on the metabolic and rheological properties of lyophilized red blood cells relative to fresh control cells collected from the same donor. The key issues that were addressed in this study for lyophilized red cell storage were:

1. Do lyophilized RBC remain metabolically viable and maintain normal cell properties upon rehydration and hence offer the potential of efficacious oxygen delivery ?
2. Can reconstituted, lyophilized red cells maintain normal metabolic and membrane properties after rehydration and storage in standard blood bank preservatives at +4C ?

In vivo survival studies of stored RBC is the final and most important criterion of the suitability of a proposed technic for extended preservation of human RBC. However, several specific in vitro parameters should be followed. The in vitro parameters used in this study to assess the quality of the lyophilized RBC included the following:

- (1) measurement of cellular levels of nucleotides (adenosine 5'triphosphate, ATP; adenosine 5' diphosphate, ADP; adenosine 5' monophosphate, AMP; 2,3-diphosphoglycerate, 2,3-DPG and lactate;
- (2) measurement of cell indices (mean cell volume (MCV), mean cell hemoglobin (MCH), mean cell hemoglobin concentration (MCHC); (3) osmotic stability curves ; (4) density distribution profiles; (5) osmotic deformability profiles from ektacytometry; and (6) filterability of RBC through 5 micron filters.

The results presented in this progress report show that reconstituted, lyophilized human red blood cells maintained in vitro cell indices that are either equal to or better than that of blood stored in CPDA according to standard blood bank procedures for comparable period.

MATERIALS AND METHODS

Six units (each unit was about 250 ml) of packed red blood cells in citrate-phosphate-dextrose-adenine were obtained from San Diego Blood Bank (San Diego, CA). Note that the RBC were stored in CPDA according to standard blood bank practices. Since the RBC were obtained in CPDA it was not necessary to resuspend the cells in our preservatives (ADSOL and AS). About 30 ml aliquots were removed from each unit and then stored at +4C. The remaining blood samples were washed in isotonic dextrose saline according to standard washing procedures [7] using an automated cell washer (Model 2991, COBE, Lakewood, CO). The washed RBC were mixed with Cryopharm's lyophilization buffers and then freeze-dried in a conventional pharmaceutical shelf freeze-dryer (Virtis Company, Gardiner, N.Y.) according to procedures developed by Cryopharm. The units of blood were lyophilized within 2 days of collection. At the end of the lyophilization cycles the samples were removed from the lyophilizer and then stored at -20C for 7 days. The lyophilized samples were rehydrated on day 7 and then washed in phosphate buffered dextrose saline (PBDS, pH 7.4 and 298mOsmol) according to standard washing procedures using an automated cell washer (Model 2991, COBE, Lakewood, CO). Samples from reconstituted RBC were assayed for glycolytic intermediates (ATP, ADP, AMP, 2,3-DPG, lactate) using neutralized perchloric acid extracts as described previously [8,9]. Osmotic fragility measurements were performed using a series of dilutions of isotonic sodium chloride solutions with water to give solutions with different degrees of hypotonicity according to a previously described method [10]. Alterations in the RBC water contents were assessed by determining changes in cell density distributions [11].

In the present study we evaluated in detail the rheological and osmotic behavior of our reconstituted lyophilized samples using the Osmotic-Gradient Ektacytometer. The ektacytometer is a highly sensitive visco-diffractometric system designed to preferentially examine the functional properties of RBC [12]. To complement the above measurements we used a published "gravity-driven" filtration assay [13] to evaluate the ability of reconstituted, lyophilized red cells to pass through a 5 micron pore filter which mimics conditions encountered in the microvasculature. For the filtration assays, diluted red cell suspensions (10^4 RBC/ μ L) are passed through a 5 micron pore filter. Results are expressed as a "Relative Filtration Index" (RFI). RFI is the ratio of the volume of RBC suspension filtered at 30 seconds over the volume of cell-free suspending medium filtered at 30 seconds.

Cell indices such as mean cell volume (MCV), mean cell hemoglobin (MCH), and mean cell hemoglobin concentration (MCHC) were measured using a standard hematology analyzer (System 9000, Serono-Baker Diagnostics Inc., Allentown, PA). Control non-lyophilized samples that had been stored in CPDA for 7 days at +4C were treated similarly to reconstituted lyophilized RBC with respect to washing. Red cell hemoglobin species were measured according to a previously described method [14].

The remaining washed reconstituted, lyophilized RBC were stored for 7 days at +4C in either ADSOL, CPDA or a special additive solution (AS) containing glucose, adenine, mannitol, phosphates.

and citrate (see table 1 for composition). The final hematocrit of all the RBC suspensions was about 52%. All of the above preservative solutions were supplemented with 100units/mL penicillin, 100ug/mL streptomycin and 25ug/mL fungizone to prevent bacterial and fungal contamination. Preservative solutions were sterilized by passing through 0.20micron filters. The pH and osmolality of the above solutions were measured with a vapor pressure osmometer (Wescor 500, Wescor, Logan, UT) at $22\pm 2^{\circ}\text{C}$. At the end of the storage period, 200uL were removed from each preparation for hemoglobin recovery according to established procedures [14] and the remaining RBC were washed in PBDS by centrifugation at 1000g for 5 minutes (Mistral 3000i Centrifuge, Fisons Scientific Equipment, Houston, TX). The washed RBC were assayed for all the parameters mentioned above. Control samples that had been stored at $+4^{\circ}\text{C}$ for the same duration were similarly treated with respect to washing. Sterility testing was performed on the samples at the end of storage. The supernatants were cultured in fluid thioglycollate medium and trypticase soy broth (Becton Dickinson and Co., Cockeysville, MD) for one week in order to check for both anaerobes and aerobes .

Statistical Analysis: Differences between lyophilized and non-lyophilized samples were analyzed with two tailed Student's t-test for paired data [15]

RESULTS FROM REFRIGERATED STORAGE STABILITY EXPERIMENTS

The formulations of the various preservatives used to store the rehydrated lyophilized RBC are shown in Table 9. These formulations are similar to those that have been used by other investigators to store RBC for extended periods. The use of phosphates in AS is important for the maintenance of intracellular pH in stored RBC. Several studies by other investigators have shown that high pH is associated with increased concentrations of glycolytic intermediates, especially ATP and 2,3-DPG. Note also that the osmolality of AS buffer is much higher than ADSOL and CPDA. The effectiveness of AS in preserving RBC during storage is believed to be due in part to the high pH, (Table 9) [16,17].

Rehydrated Lyophilized RBC and Control RBC Stored in CPDA: In this study the lyophilized RBC were stored at -20C for 7 days and then rehydrated according to procedures developed by Cryopharm. Upon rehydration, lyophilized RBC maintained cell indices that were either equal to or better than those obtained with control non-lyophilized RBC that had been stored for a comparable period in the liquid state at +4C according to standard blood bank procedures. The maximum age of the RBC at this stage was 9 days, because the RBC were 2 days old prior to lyophilization. The levels of glycolytic intermediates, ATP and 2,3-DPG, in lyophilized RBC are not significantly different from control cells, $p > 0.05$, (Table 10). Note however, that the data show a trend towards higher levels of these intermediates in reconstituted, lyophilized RBC. The lack of significance at 0.05 level may be due in part to the relatively small sample size. Overall glycolysis was assessed by the amount of lactate formed. Control RBC contained significantly higher levels of lactate than reconstituted, lyophilized RBC, $p < 0.001$, (Table 10). The lower levels of lactate in lyophilized RBC suggest that there was little glycolytic activity during storage in the lyophilized state. When osmotic fragility was examined in washed red cell suspensions from both lyophilized and non-lyophilized control RBC, there was no significant difference between the two cell types, $p > 0.05$, (Figure 5). Increased intracellular concentration of lactate is associated with an increase in cell lysis (i.e., increase in osmotic fragility). The lack of difference in osmotic fragility between lyophilized and non-lyophilized RBC may be due to the fact that the osmotic fragility tests were performed after 30 minutes of equilibration in isotonic saline. During this incubation period most of the accumulated lactate may have leaked out of the cells. Indirect evidence for the effect of increased lactate concentration is indicated by the slight increase in MCV of control RBC, (Table 10). One can not rule out the possibility that the observed increase in MCV of stored RBC was due to accumulation of other osmotically active solutes within the RBC [10].

Red cell biophysical properties were evaluated with the ektacytometer. The ektacytometer combines deformation of RBC by hydrodynamic shear forces with laser diffraction to detect the average deformability (elongation) of RBC. The resulting deformation of the RBC is seen as an elongation of the cell and is expressed as the "Maximum Deformability Index" (DI_{max}). DI_{max} is traced on an X-Y recorder as a function of the suspending medium osmolality. Variations in the suspending medium

osmolality and applied shear stress allow detailed evaluation of the different factors regulating cell deformability. The osmotic deformability profiles obtained from this assay provide information about cell water content, the relationship between surface area and cell volume, and heterogeneity in these cellular properties. For example, cells with reduced surface area to volume ratio (S/V) deform less extensively than normal cells as seen by a pronounced decrease in DI_{max} . Cells with increased membrane rigidity are also less deformable than normal. Unlike ektacytometry measurements, osmotic fragility of RBC is measured in the absence of any hydrodynamic shear stress on the RBC. Osmotic fragility provides an estimate of the relationship between surface area and volume as it exists when the RBC are suspended in salt solutions with different osmolalities. Increase in osmotic fragility is usually associated with hemolysis of RBC even when suspended in isotonic saline. Measurement of whole cell deformability at different osmolalities with the Osmotic Gradient Ektacytometer showed that the osmotic deformability profiles and maximum cell elongation of reconstituted lyophilized RBC are not significantly different from control RBC. Normal deformability profiles of lyophilized RBC demonstrate that the normal relationship between cell volume and surface area was not altered during the lyophilization process. In spite of the removal of about 80% of available water from the RBC suspension during lyophilization, there was no indication of any oxidation or dehydration induced damage to the RBC hemoglobin as judged by the low levels of methemoglobin and hemichromes (denatured hemoglobin) in reconstituted lyophilized RBC, (Table 10). Formation of high concentrations of both methemoglobin and hemichrome have been found in lyophilized hemoglobin preparations [18].

Refrigerated storage of rehydrated lyophilized RBC and control RBC stored in CPDA: The results in Table 11 show that reconstituted, lyophilized RBC that had been stored in ADSOL and AS for 7 days at +4C maintained levels of ATP and 2,3-DPG that were significantly higher than those obtained with control RBC stored in CPDA under similar conditions for a comparable period. The levels of these glycolytic intermediates in reconstituted lyophilized RBC that were stored in CPDA were not significantly different from control RBC. During the 7 day storage at +4C, all RBC underwent progressive increase in the levels of lactate indicating that glycolysis was functioning under these conditions of storage. The increase observed in control RBC stored in CPDA was significantly higher than those obtained with reconstituted, lyophilized RBC that were stored in either CPDA, ADSOL or AS. This increase in intracellular concentration of lactate was accompanied by significant increase in mean cell volume (MCV) and increase in osmotic fragility of control RBC when compared with reconstituted, lyophilized RBC. Representative osmotic fragility curves of the various red cell preparations are shown in Figure 5.

The relationship between the deformability of RBC and suspending medium osmolality for the different red cell preparations is shown in Figures 6A-6D. The most important features of these deformability profiles are : (1) DI_{max} , which is the maximum cell deformation at isotonicity, was

slightly decreased in reconstituted, lyophilized RBC. The DI_{max} represents a balance in the adjustment of the surface area to volume ratio and the intracellular viscosity of the cell. Note also that there was also a decrease in the filterability of CPDA and ADSOL-stored lyophilized RBC when compared to control RBC that had been stored for comparable period in CPDA, (Table 11); (2) Osmolality at which DI reaches a minimum (O). This decrease in the ability of the cell to deform results from the increase in the volume of the cell at a constant surface area. The hypotonic osmolality at which DI reached a minimum coincided with the osmolality at which 50% of the RBC hemolyzed in the osmotic fragility assay, Figure 5, and is between 150-170mOsmol for all the samples evaluated. Note that as the suspending osmolality was increased above isotonicity, the DI_{max} again decreased. This decrease in hypertonic deformability resulted from an increase in intracellular viscosity secondary to osmotic water loss and increasing hemoglobin concentration. The hypertonic osmolality at which the DI equals half of the maximum deformability gives information about the mean cell hemoglobin concentration and the level of cell dehydration. Both reconstituted, lyophilized and non-lyophilized control RBC showed similar responses in the hypertonic arm. The fact that the lyophilized RBC were not excessively dehydrated is demonstrated by normal density distribution profiles, (Figure 7). In overview, the osmotic deformability profiles of reconstituted, lyophilized RBC are very similar to control RBC.

A summary of the changes in glycolytic intermediates is shown in Table 12. Note the rapid decrease in ATP, 2,3-DPG and increase in lactate levels of control RBC as the storage duration increased from 9 to 16 days. Reconstituted, lyophilized RBC showed less rapid changes in the above metabolites when compared to control RBC. All the samples evaluated for this study remained sterile at the end of the storage period with a hemoglobin loss of less than 2%.

B. Rehydration and Washing Procedures

The main objective of our research efforts is to eventually develop a "NO-WASH" reconstitution procedure. The present research efforts are directed at simplifying the formulations of our rehydration buffers such that the lyophilized RBC can be reconstituted with standard dextrose saline. Preliminary results indicate that the present lyophilized RBC can be rehydrated with a "NO WASH" method..

C. Process Development and Container Design

The process development group is currently evaluating different container designs. Some of the materials that are being considered possess properties that will allow efficient removal of water vapor during the lyophilization cycle. In addition, this group has been using different mathematical models to determine both the storage stability of our lyophilized RBC and optimal lyophilization procedures.

FUTURE PLANS

Preliminary results from our in vivo survival studies demonstrates that red blood cells can be successfully lyophilized, rehydrated and transfused into a normal autologous donor with maintenance of normal cell indices and in vivo survival. Future studies will need to address the following key issues:

1. **Storage stability studies:** To determine the shelf life of lyophilized human red blood cells at +4C or higher. Our research activity will be directed at understanding both the biophysical and biochemical changes that limit the shelf of lyophilized RBC. Understanding of these biochemical changes will be the key to our developing processing conditions that extend the current shelf life.
2. **Improvement in sample configuration and selection of final container:** Different materials and sample configurations will be evaluated with the hope of designing a new lyophilization bag so that lyophilization can be efficiently carried out. The current blood to buffer ratio is 1:3 (i.e., 1 ml of RBC to 3 ml of buffer). We will continue to evaluate the effects of blood to buffer ratios on the overall quality of lyophilized products. Knowledge of the optimal blood to buffer ratio will guide our efforts in designing the final lyophilization bag.
3. **Simplification of the rehydration procedure** such that the lyophilized RBC can be rehydrated without additional washes.
4. **Determine the relationship between storage stability, residual moisture and in vivo survival.** Red blood cells will be lyophilized to different residual moisture and the overall cell quality will be evaluated to determine the maximum level of dryness the RBC can tolerate without any major alteration

in cell properties. In vivo survival of RBC dried to different levels of residual moisture will be determined using cynomolgus monkeys .

5. Determine the oxygen-carrying efficacy of our rehydrated, lyophilized RBC by measuring the oxygen dissociation curve .

6. Scale-up and cycle development: Currently, the present lyophilization procedures can accommodate a clinical size sample of about 160g of blood buffer mixture. We will continue to modify our lyophilization cycles such that full units of blood can be lyophilized.

7. Increase the overall recovery of reconstituted lyophilized RBC. Using small bench samples (about 10-15g blood and buffer mixture) we have been able to obtain overall recovery of at least 80%. We will design experiments to reduce the loss of cell recovery at higher sample mass.

8. Begin to evaluate issues related to the toxicity and safety issues of the various components of our buffer formulations in preparation for filing of IND at the end of the year

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TABLE 1: CRYOPHARM CORPORATION RESEARCH MILESTONE CHART-1989

CRYOPHARM CORPORATION
RESEARCH MILESTONES CHART
FREEZE-DRIED RED CELLS

<u>Project Activities</u>	<u>Current Status</u>	<u>Milestone</u>	<u>Projected Start</u>	<u>Projected Completion</u>
Define Shelf Lyophilization Parameters: Define optimal temperature, pressure conditions. Evaluate sample configuration.	No defined cycle	Defined cycle worked-out	Year 1	Year 1
Evaluate Existing Reconstitution Protocol: Mixing and temperature conditions.	~70% initial yield	>80% initial yield	Year 1	Year 1
Optimize Product Properties: Cell yield (at infusion stage). Residual moisture (in dry state). Final product sterility (at infusion stage). Shelf Life: Refrigerated storage. Room temperature storage.	-35-40% -3% Not done >10 months ~2 weeks	>50% -1% Demonstrated >2 years 1-2 months	Year 1 Year 1 Year 1 Year 1 Year 1	Year 2 Year 2 Year 2 Year 3 Year 3
Evaluation of Enzyme Converted Red Cells.	Not done	Initial tests	Year 2	Year 2
In vivo Animal Circulation Studies: Pilot studies in domestic pigs. GLP quality studies in domestic pigs.	Not done Not done	Done If pilot tests successful.	Year 1 Year 2	Year 1 Year 2
In vitro Animal Red Cell Studies: (Survey models if pig cells do not circulate)	Preliminary data in.	More samples for FDA.	Year 2	Year 2
Plastic Container Development.	First prototype	Developed.	Year 1	Year 1
Streamline Reconstitution and Washes.	Not done	Underway	Year 3	To be deter.
Phase I Clinical Trials of Lyophilized Cells.	Not done	File IND	Year 3	Continues...

**TABLE 2: IN VITRO CELL INDICES OF RECONSTITUTED LYOPHILIZED RBC FROM
CYNOMOLGUS MONKEY**

In Vitro Cell Parameters	Fresh RBC from Cynomolgus Monkey	Lyophilized RBC from Monkey # 344	Lyophilized RBC from Monkey # 316
Overall Cell Recovery (%)	-	68.7	71.1
Osmotic Stability (%)	95-100	93.4	92.0
Mean Cell Volume (fl)	66.7 + 1.7	70.9	67.2
Mean Cell Hemoglobin (pg)	20.2 + 0.4	21.2	20.3
Mean Cell Hemoglobin Concentration (g/dL)	30.3 + 0.4	29.9	30.1
Maximum Cell Deformability (DImax)	0.59-0.600	0.552	0.569
% Control DImax	100	95.3	100.0
Osmotic Deformability Profile	Normal	Normal	Normal

TABLE 3A: POST-INFUSION RADIOACTIVITY OF CHROMIUM LABELLED RECONSTITUTED, LYOPHILIZED RBC FROM CYNOMOLGUS MONKEY #316

Time Points	Time Counted	Total Counts**	Counts Per Minute (CPM)	CPM/ml of whole blood	% Survival of Transfused RBC	% Survival for fresh non-lyophilized RBC
0	1 minute	10187	10187	50935	100	100.0
5 minutes	1 minute	10175	10175	50875	99.9	98.8
7.5 minutes	1 minute	9845	9845	49225	96.6	ND
10 minutes	1 minute	9347	9347	46735	91.8	97.6
15 minutes	1 minute	9726	9726	48630	95.5	96.4
30 minutes	1 minute	8199	8199	40995	80.5	97.0
1 hour	1 minute	10650	10650	53250	100.0	96.1
2 hours	1 minute	11680	11680	58400	100.0	100.0
4 hours	1 minute	10420	10420	52100	100.0	100.0
8 hours	1 minute	10211	10211	51055	100.0	ND
12 hours	1 minute	9960	9960	49800	97.8	ND
24 hours	1 minute	8863*	8863	44315	87.0	88.4
48 hours	1 minute	7915*	7915	39575	77.7	ND
72 hours	1 minute	6954*	6954	34770	68.3	ND
96 hours	1 minute	6619*	6619	33095	65.0	ND
120 hours	1 minute	6531*	6531	32655	64.1	ND
144 hours	1 minute	5531*	5531	27655	54.3	ND

* Radioactive counts are corrected for elution of chromium according to standard procedures for estimating in vivo survival of transfused human red blood cells []. ** Total counts have been corrected for background.

TABLE 3B: POST-INFUSION RADIOACTIVITY OF CHROMIUM LABELLED RECONSTITUTED, LYOPHILIZED RBC FROM CYNOMOLGUS MONKEY #344

Time Points	Time Counted	Total counts**	Counts Per Minute (CPM)	CPM/ml of whole blood	% Survival of Transfused RBC	% Survival for fresh non-lyophilized RBC
0	1 minute	17267	17267	86335	100.0	100.0
5 minutes	1 minute	16824	16824	84120	97.4	98.8
7.5 minutes	1 minute	16867	16867	84335	97.7	ND
10 minutes	1 minute	15925	15925	79625	92.2	97.6
15 minutes	1 minute	16059	16059	80295	93.0	96.4
30 minutes	1 minute	15876	15876	79380	92.0	97.0
1 hour	1 minute	17527	17527	87635	100.0	96.1
2 hours	1 minute	15378	15378	76890	89.1	100.0
4 hours	1 minute	18208	18208	91040	100.0	100.0
8 hours	1 minute	16959	16959	84795	98.2	ND
12 hours	1 minute	16867	16867	84335	97.7	ND
24 hours	1 minute	16147*	16147	80735	93.5	88.4
48 hours	1 minute	10075*	10075	50375	58.4	ND
72 hours	1 minute	12027*	12027	60135	69.7	ND
96 hours	1 minute	11688*	11688	58440	67.7	ND
120 hours	1 minute	10367*	10367	51835	60.0	ND
144 hours	1 minute	10030*	10030	50150	58.1	ND

Abbreviation: ND, Not Determined

* Radioactive counts are corrected for elution of chromium according to standard procedures for estimating in vivo survival of transfused red blood cells** Total counts have been corrected for background.

TABLE 4: IN VITRO PARAMETERS FOR RECONSTITUTED, LYOPHILIZED HUMAN RED BLOOD CELLS

CELL PARAMETERS	Fresh Non-Lyophilized Red Blood Cells	Reconstituted lyophilized RBC from Subject # 1
Osmotic Stability (%)	95-100	98.3
Mean Cell Volume (fl)	80-100	91.3
Mean Cell Hemoglobin (pg)	25-35	29.4
Mean Cell Hemoglobin Concentration (g/dL)	31-37	32.3
Maximum Cell Deformability (D _{max})	0.500-0.670	0.597
% Control D _{max}	100.0	95.7
Osmotic deformability profile	Normal	Normal
Relative Filterability (RFI)	1.00	0.885
% Control RFI	100	88.5

TABLE 5: POST-INFUSION RADIOACTIVITY OF CHROMIUM LABELLED RECONSTITUTED, LYOPHILIZED HUMAN RED BLOOD CELLS

Time Points after infusion	Total Time Counted	Counts Per Minute (CPM)	HCT (%)	CPM/ mL of RBC	% Survival of Transfused RBC (Ext To)	%Survival of Transfused RBC (Th To)
5 minutes	30 minutes	2742	43.0	6377	99.1	98.7
7.5 minutes	30 minutes	2767	43.5	6361	98.9	98.4
12 minutes	30 minutes	2716	43.0	6316	98.2	97.7
15 minutes	30 minutes	2701	43.0	6281	97.7	97.2
20 minutes	30 minutes	2600	44.0	6091	94.7	94.3
30 minutes	30 minutes	2666	42.5	6273	97.5	97.1
45 minutes	30 minutes	2575	42.5	6059	94.2	93.8
1 hour	30 minutes	2622	43.0	6098	94.8	94.4
2 hours	30 minutes	2593	43.0	6030	93.8	93.3
4 hours	30 minutes	2496	43.0	5805	90.3	89.8
6 hours	30 minutes	2474	43.0	5753	89.4	89.0
24 hours	30 minutes	2379	43.0	5533	86.0	85.6
48 hours	30 minutes	2347	43.0	5458	84.9	84.5
72 hours	30 minutes	2216	43.0	5153	80.1	79.7
96 hours	30 minutes	2145	43.0	4988	77.5	77.2

Abbreviations: Ext To, Extrapolated Time Zero; Th To, Theoretical Time Zero.

** Total counts have been corrected for background.

TABLE 6: MEASUREMENT OF DISTRIBUTION OF RADIOLABELLED RECONSTITUTED, LYOPHILIZED RBC USING EXTERNAL UPTAKE PROBE

TIME POINTS	HEART	LIVER	SPLEEN	SPLEEN/ HEART	LIVER/ HEART	SPLEEN/ LIVER
4 Hours	28250	19133	23825	0.8	0.7	23825
24 Hours	25021	18341	23733	1.0	0.7	23733
% of Total injected dose at 4 hours post-infusion	39.7	26.9	33.5	0.8	0.7	33.5
% of Total injected dose at 24 hours post-infusion	37.3	27.3	35.4	1.0	0.7	35.4

TABLE 7: LEVELS OF RADIOACTIVITY EXCRETED IN URINE FROM HUMAN VOLUNTEER

TIME POINTS	VOLUME OF URINE (mL)	CPM/mL	CPM/mL
0 - 6 Hours	300	782	234,450
6 - 12 Hours	200	1070	213,900
12 - 18 Hours	0	0	0
18 - 24 Hours	470	478	224,425
% Injected Dose Excreted in Urine	-	-	4.1

** Total counts have been corrected for background.

NOTE: % Injected Dose Excreted in Urine = $\frac{\text{Total Overall Urine CPM} \times 100}{\text{Injected Dose}}$

TABLE 8: POST-TRANSFUSION VITAL SIGNS OF SUBJECT # 1

Post-Transfusion Time points	Temperature (degree F)	Pulse per Minute	Respirations (cycles per minute)	Blood Pressure (mmHg)
1 hour	97	72	18	104/76
2 hours	98	72	16	100/80
3 hours	98	84	20	98/70
4 hours	99	80	20	90/50
24 hours	98	80	20	100/70

Table9 : Compositions of common blood bank preservatives used to store the reconstituted lyophilized RBC at +4C for 7 days .

FORMULATIONS	ADSOL	AS	CPD-A
Glucose (mM)	121.0	139.0	177.0
Na Citrate (mM)	-	33.3	89.0
Na ₂ HPO ₄ (mM)	-	12.0	-
NaH ₂ PO ₄ (mM)	-	2.9	16.0
Adenine (mM)	2.0	2.0	2.0
NaCl (mM)	154.0	-	-
Mannitol (mM)	41.0	41.0	-
Citric acid (mM)	-	-	17.0
pH	6.3	9.0	5.6
Osmolality (mOsmol/kg H ₂ O)	406.0	330.0	494.0

Abbreviations: CPDA, Citrate Phosphate Dextrose Adenine; AS, Adenine Special buffer.

Both ADSOL and CPDA are standard preservative solutions used by most blood bank facilities for extended storage of human red blood cells in the liquid state at +4C. ADSOL has been shown by several investigators to be more effective in preventing excessive loss of both ATP and 2,3-DPG during storage . AS is an experimental formulation developed by the American Red Cross and is believed to be far superior to both CPDA and ADSOL in maintaining the integrity of the RBC during storage. Note that unlike ADSOL and CPDA , AS contained phosphates which are included to prevent excessive decrease in intracellular pH of RBC during storage. Note also that the pH of AS is much higher than that for ADSOL and CPDA and is generally believed to be one of the main factors for the effectiveness of AS as a preservative.

Table 10: Human red blood cells (RBC) were lyophilized and then stored at -20C for 7 days prior to rehydration. Note that the RBC used for this study were stored in CPDA for 2 days at +4C prior to lyophilization.

Assays	Freshly Drawn RBC	Control RBC Stored in CPDA	Rehydrated lyophilized RBC	P*
ATP ($\mu\text{mol/mL}$ RBC)	1.44	1.33 \pm 0.15	1.45 \pm 0.18	NS
ADP ($\mu\text{mol/mL}$ RBC)	0.21	0.0	0.0	NS
AMP ($\mu\text{mol/ml}$ RBC)	0.02	0.0	0.0	NS
2,3-DPG ($\mu\text{mol/mL}$ RBC)	4.17	1.04 \pm 0.67	1.63 \pm 0.94	NS
Lactate ($\mu\text{mol/mL}$ RBC)	2.07	15.2 \pm 0.49	1.28 \pm 0.45	p<0.001
Density (g/mL)	1.10	1.096 \pm 0.002	1.100 \pm 0.002	p<0.02
Osmotic Fragility (%)	0-5	4.5 \pm 1.1	5.4 \pm 1.2	NS
MCV (fl)	80-100	93.5 \pm 4.41	90.8 \pm 4.4	NS
MCH (pg)	25-35	31.0 \pm 1.8	29.9 \pm 1.6	NS
MCHC (g/dL)	31-37	33.1 \pm 0.5	33.0 \pm 1.1	NS
Oxy-hemoglobin (%)	100.0	99.1 \pm 0.8	97.385 \pm 1.832	NS
Met-hemoglobin (%)	0.0	0.0	0.073 \pm 0.180	NS
Hemichrome (%)	0.0	0.85 \pm 0.81	2.54 \pm 1.68	NS
Maximum Deformability Index (DI _{max})	0.64-0.68	0.69 \pm 0.02	0.64 \pm 0.02	NS
Relative Filtration Index (RFI)	0.90-1.0	0.92 \pm 0.09	0.70 \pm 0.16	p<0.02

Data represent the mean \pm SD for six samples . Values for freshly drawn RBC are included for comparison with present data. Abbreviations: ATP, adenosine 5'triphosphate; ADP, adenosine 5' diphosphate; AMP, adenosine 5' monophosphate; 2,3-DPG, 2,3-diphosphoglycerate; MCV, Mean Cell Volume; fl, femtoliters; MCH, Mean Cell Hemoglobin; pg, picograms; MCHC, Mean Cell Hemoglobin Concentration; g/dL, grams per deciliter; NS, not significant (p> 0.05). Note that the maximum deformability of the RBC was obtained at a constant shear stress of about 230 dynes/cm² .

* Differences between non-lyophilized and rehydrated lyophilized red blood cells.

Table 11: Comparison of the levels of glycolytic intermediates and cell indices of rehydrated lyophilized red blood cells and control normal non-lyophilized red blood cells. Note that both rehydrated lyophilized and non-lyophilized red blood cells were stored at +4C at for 7 days prior to measurement of cell indices. The control cell has aged 16 days.

Assays	Freshly drawn RBC	Control RBC Stored CPDA	Lyo-RBC in ADSOL	Lyo-RBC in AS	Lyo-RBC in CPDA
ATP ($\mu\text{mol/mL RBC}$)	1.44	1.16 \pm 0.13	1.57 \pm 0.18 p<0.001	1.63 \pm 0.31 p<0.008	1.17 \pm 0.69 NS
ADP ($\mu\text{mol/mL RBC}$)	0.21	0.021 \pm 0.015	0.002 \pm 0.003 p<0.05	0.0 NS	0.0
AMP ($\mu\text{mol/mL RBC}$)	0.020	0.0	0.017 \pm 0.019 NS	0.0 NS	0.0 NS
2,3-DPG ($\mu\text{mol/mL RBC}$)	4.17	0.23 \pm 0.24	1.64 \pm 0.90 p<0.004	2.21 \pm 1.09 p<0.008	0.14 \pm 0.17 NS
Lactate ($\mu\text{mol/mL RBC}$)	2.07	27.6 \pm 3.2	8.2 \pm 3.08 p<0.0001	10.0 \pm 1.7** p<0.001	6.2 \pm 4.8 p<0.001
Density (g/mL)	1.090-1.100	1.095 \pm 0.003	1.100 \pm 0.003	1.098 \pm 0.002	1.106 \pm 0.004
Osmotic Fragility (%)	0-5	10.5 \pm 3.0	5.3 \pm 0.7 p<0.007	6.2 \pm 0.4 NS	8.1 \pm 2.0 NS
MCV (fl)	80-100	95.3 \pm 4.7	93.2 \pm 4.6 p<0.001	88.6 \pm 4.4 p<0.001	90.3 \pm 4.9 p<0.025
MCH (pg)	25-35	31.3 \pm 1.6	30.5 \pm 1.6 p<0.025	30.4 \pm 2.1 NS	31.3 \pm 2.2 NS
MCHC (g/dL)	31-37	32.9 \pm 0.4	32.7 \pm 0.5 NS	34.4 \pm 0.8 p<0.002	34.7 \pm 1.1 p<0.015
Oxy-hemoglobin (%)	95-100	99.8 \pm 0.3	97.2 \pm 1.5 p<0.011	98.7 \pm 1.2 NS	99.5 \pm 0.6 NS
Met-hemoglobin (%)	0-0.50	0.19 \pm 0.3	0.29 \pm 0.68 NS	0.09 \pm 0.21 NS	0.44 \pm 0.60 NS
Hemichrome (%)	0.0	0.0	2.54 \pm 1.24 NS	1.17 \pm 1.22 NS	0.0 NS
Hemoglobin Recovery (%)	95-100	100.0	98.7 \pm 1.0 NS	99.3 \pm 0.3 NS	99.2 \pm 0.3 NS
Deformability Index (DI _{max})	0.64-0.68	0.65 \pm 0.02	0.63 \pm 0.02 p<0.005	0.61 \pm 0.03 p<0.029	0.61 \pm 0.03 p<0.007
Relative Filtration Index (RFI)	0.90-1.0	0.99 \pm 0.02	0.85 \pm 0.06 p<0.003	0.95 \pm 0.04 NS	0.86 \pm 0.08 NS
Number of Samples		n=6	n=6	n=5	n=4

Data represent the mean \pm SD for 4-6 samples . Values for normal RBC are included for comparison with present data. Abbreviations: NS, not significant, p>0.05; ATP, adenosine 5'triphosphate; ADP, adenosine 5' diphosphate; AMP, adenosine 5' monophosphate; 2,3-DPG, 2,3-diphosphoglycerate; MCV, Mean Cell Volume; MCH, Mean Cell Hemoglobin; MCHC, Mean Cell Hemoglobin Concentrations. ** Lactate levels were measured in 4 samples only .

Table 12: Levels of glycolytic intermediates in lyophilized and control RBC. All samples were stored at +4C for 2 days in CPDA prior to receipt at Cryopharm and initiation of study.

	Control RBC Stored in CPDA	Stored Lyo RBC	Control RBC Stored in CPDA	Lyo RBC Stored in ADSOL	Lyo RBC Stored in AS	Lyo RBC Stored in CPDA
ATP μmol/ml RBC	1.33±0.15	1.45±0.18	1.16±0.13	1.57±0.18	1.63±0.31	1.17±0.69
2,3DPG μmol/ml RBC	1.04±0.67	1.63±0.94	0.23±0.24	1.64±0.90	2.21±1.09	0.14±0.17
Lactate μmol/ml RBC	15.2±0.5	1.3±0.5	27.6±3.2	8.2±3.1	10.0±1.7	6.2±4.8
Day of Storage	9	9	16	16	16	16
Number of Samples	6	6	6	6	5	4

Data represent the mean ± SD for 4-6 samples . Normal values are included for comparison with present data. Abbreviations: NS, not significant, p>0.05; ATP, adenosine 5'triphosphate; ADP, adenosine 5' diphosphate; AMP, adenosine 5' monophosphate; 2,3-DPG, 2,3-diphosphoglycerate; Lyo, Lyophilized . Note: Stored Lyo RBC = Lyophilized RBC stored in the dried state at -20C for 7 days and then rehydrated. Stored Lyo RBC in ADSOL = Lyophilized RBC stored in the dried state for 7 days and then rehydrated. Reconstituted, lyophilized RBC were then stored in ADSOL for an additional 7 days. The total storage period was 16 days, i.e., total of 14 days in dried state + liquid state , and 2 days in CPDA (prior to receipt at Cryopharm). Stored Lyo RBC in AS = Same as for ADSOL. Stored Lyo RBC in CPDA = Same as for ADSOL.

Figure 1: In vivo survival of chromium labelled autologous red blood cells in cynomolgus monkeys. Note that both lyophilized RBC and fresh RBC have similar in vivo behavior

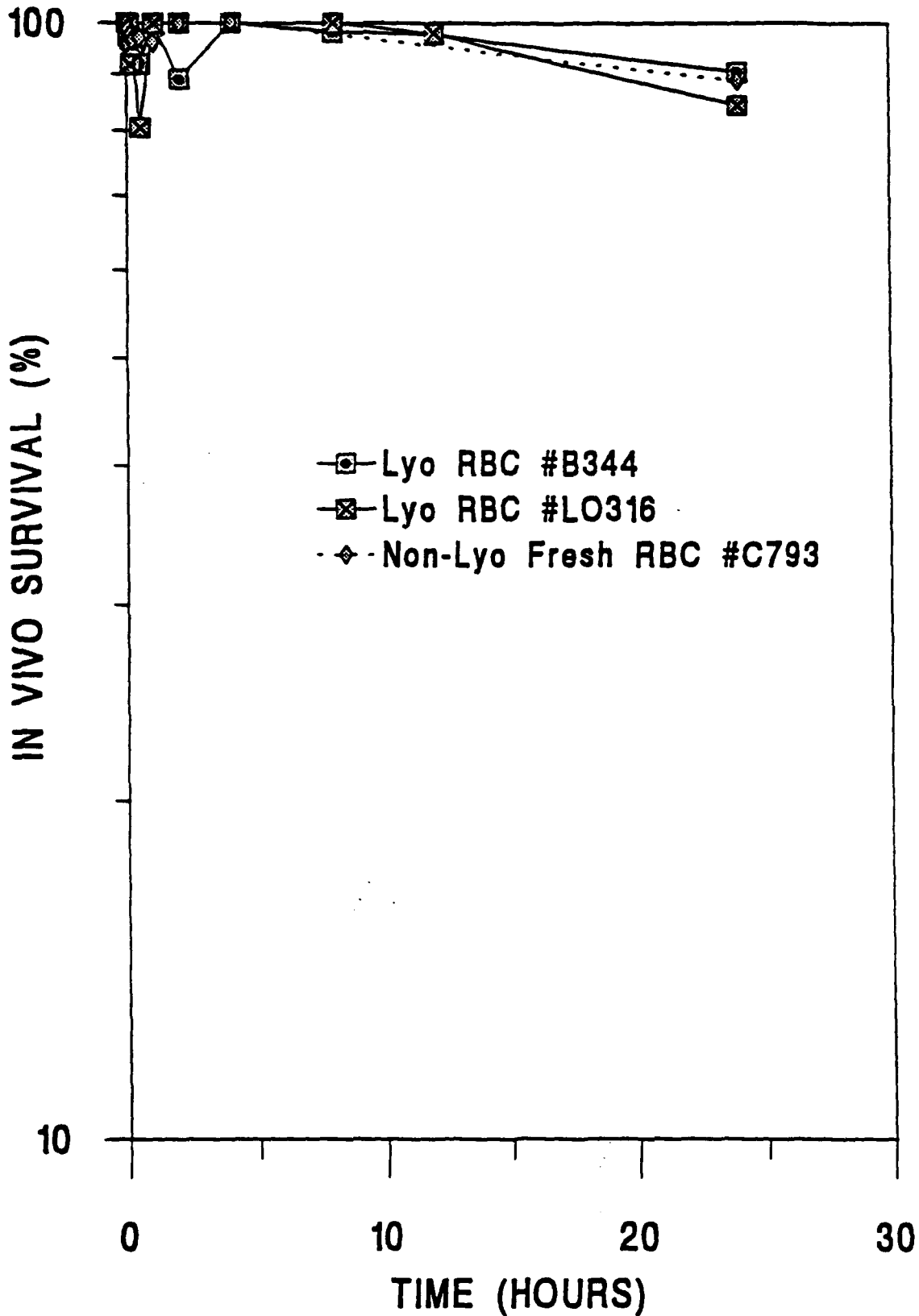


FIGURE 2A: DETERMINATION OF CIRCULATING HALF LIFE OF CHROMIUM LABELLED LYOPHILIZED AND RECONSTITUTED RED BLOOD CELLS FROM CYNOMOLGUS MONKEY

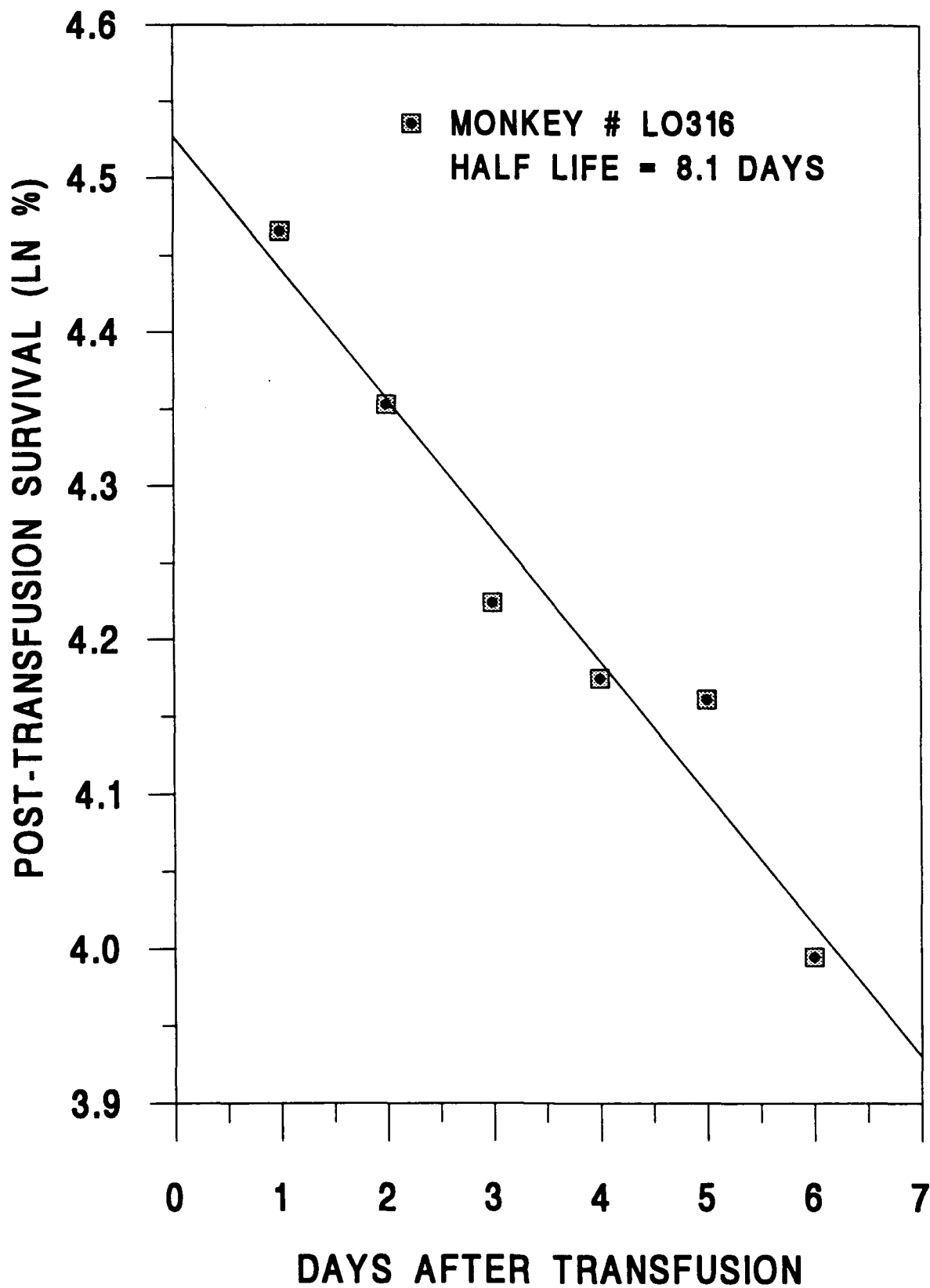


FIGURE 2B: DETERMINATION OF CIRCULATING HALF LIFE OF CHROMIUM LABELLED LYOPHILIZED AND RECONSTITUTED RED BLOOD CELLS FROM CYNOMOLGUS MONKEY

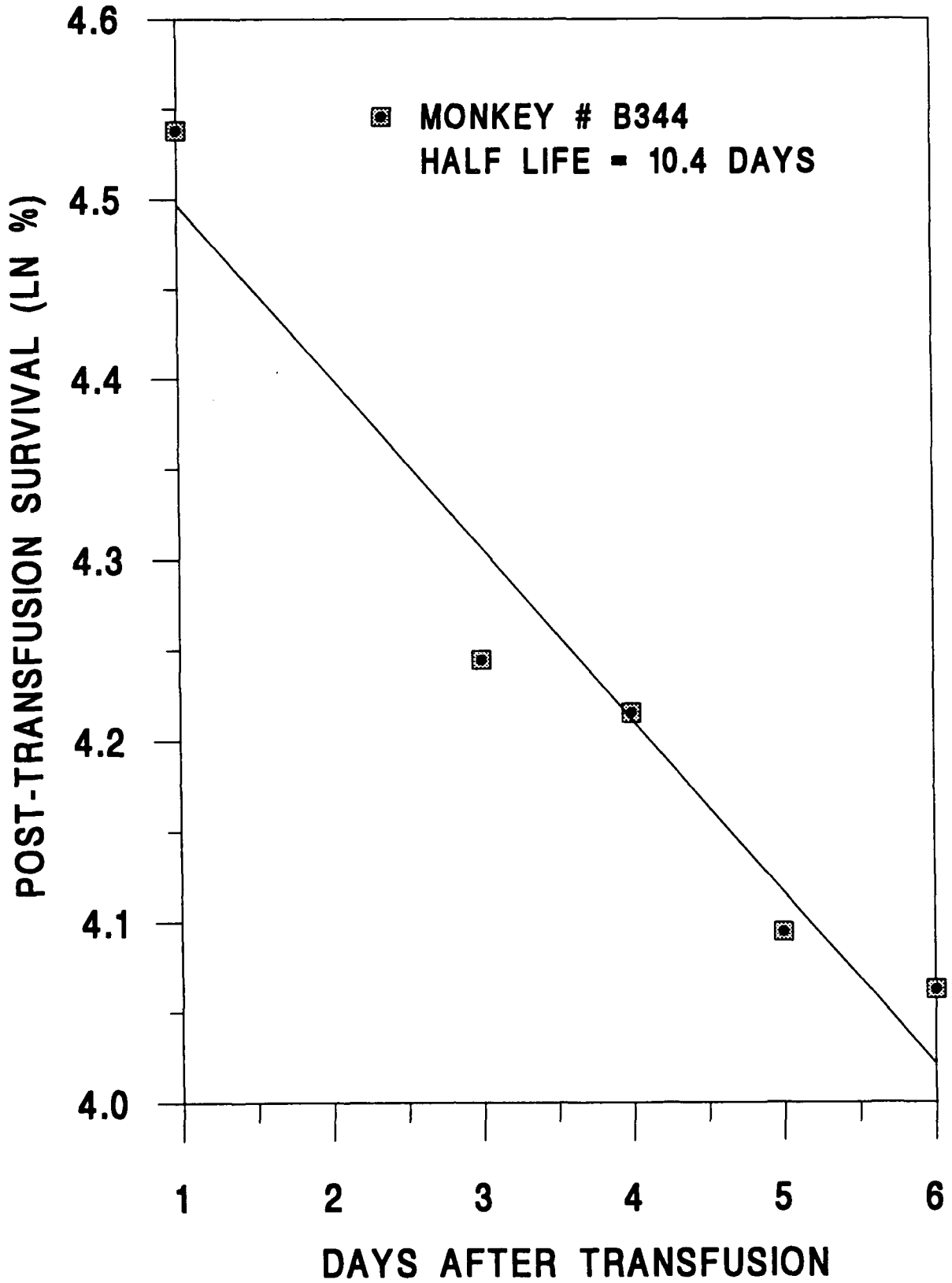


FIGURE 3: 24 HOUR POST-TRANSFUSION SURVIVAL OF LYOPHILIZED HUMAN RBC

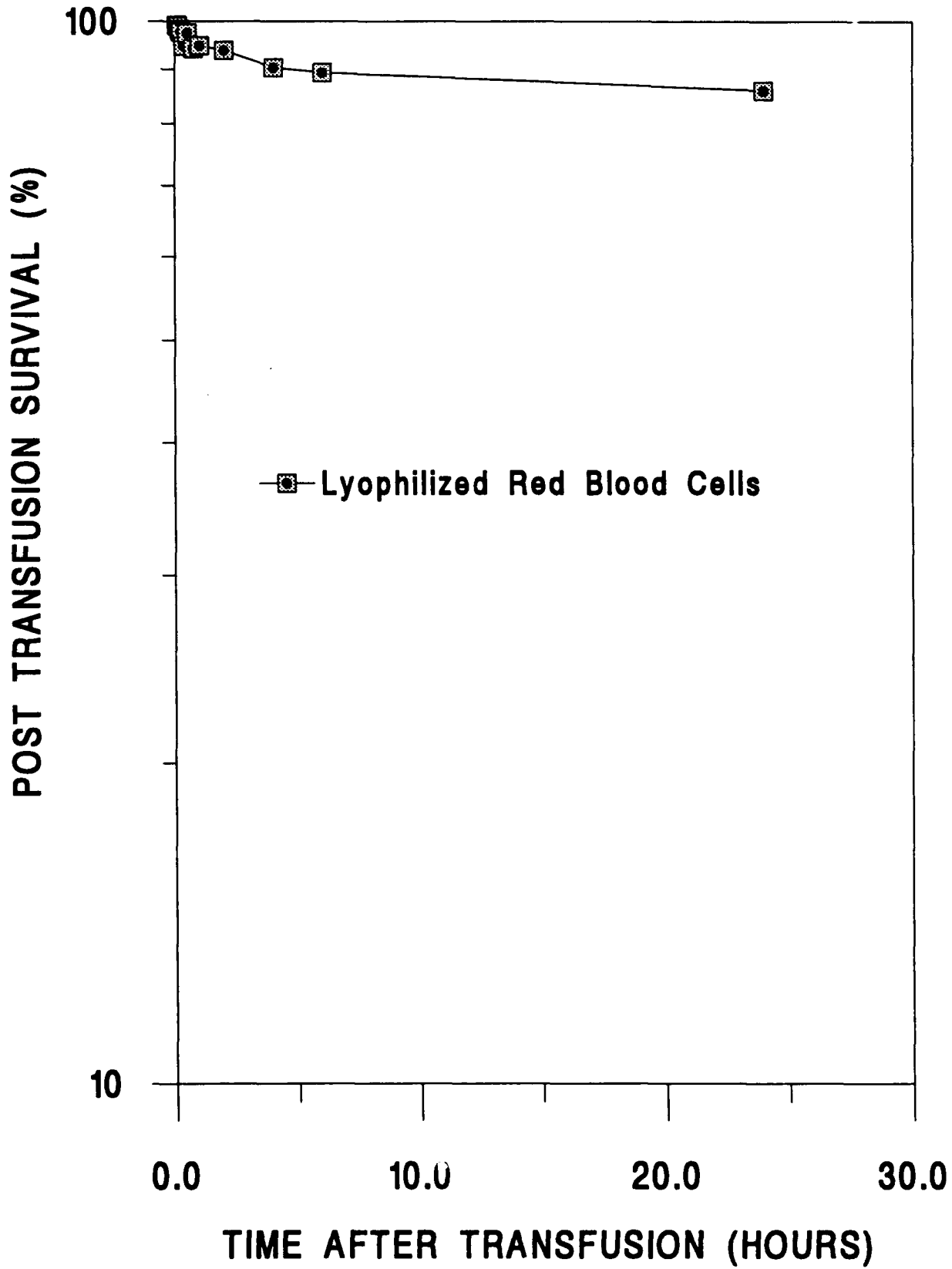
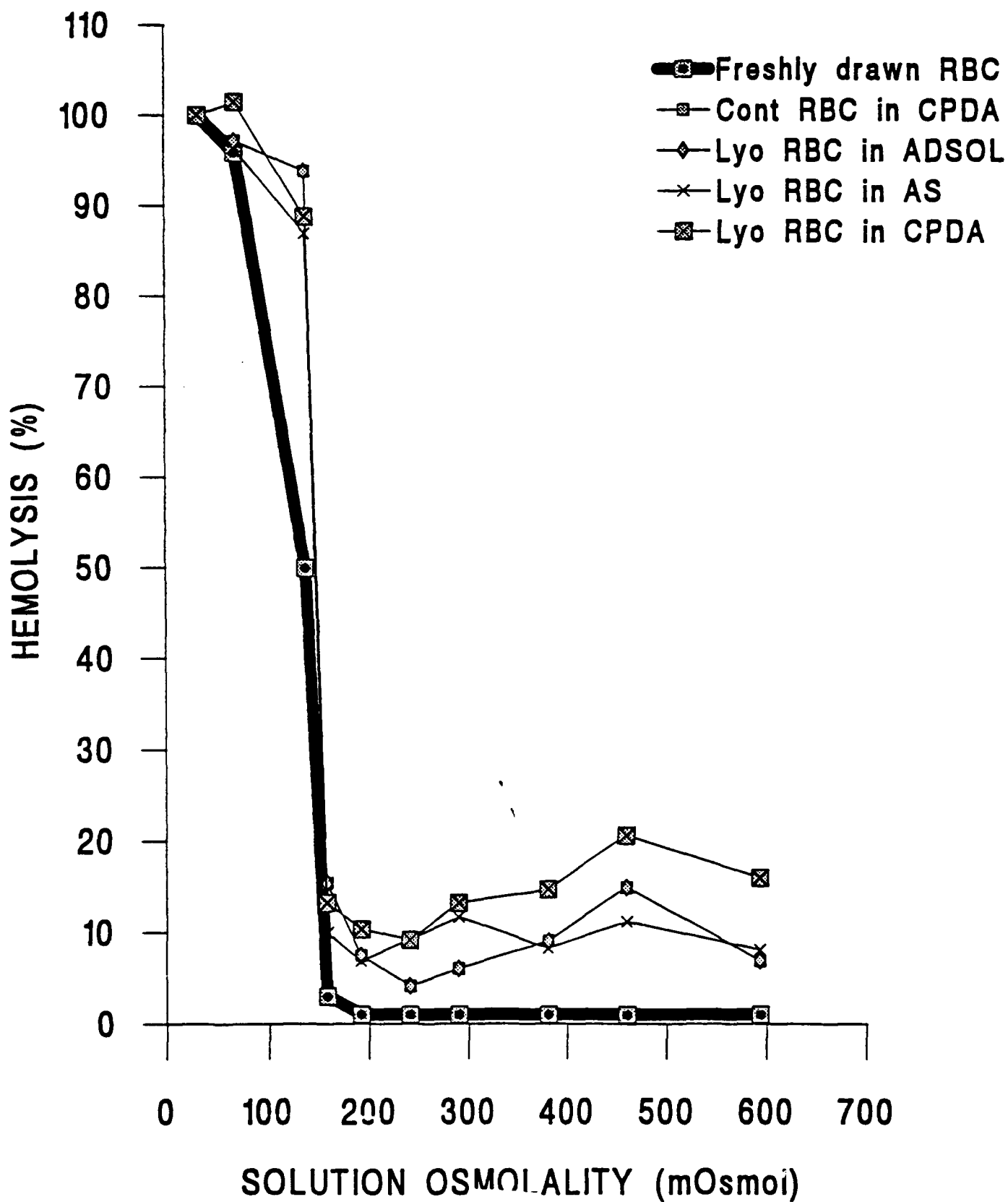
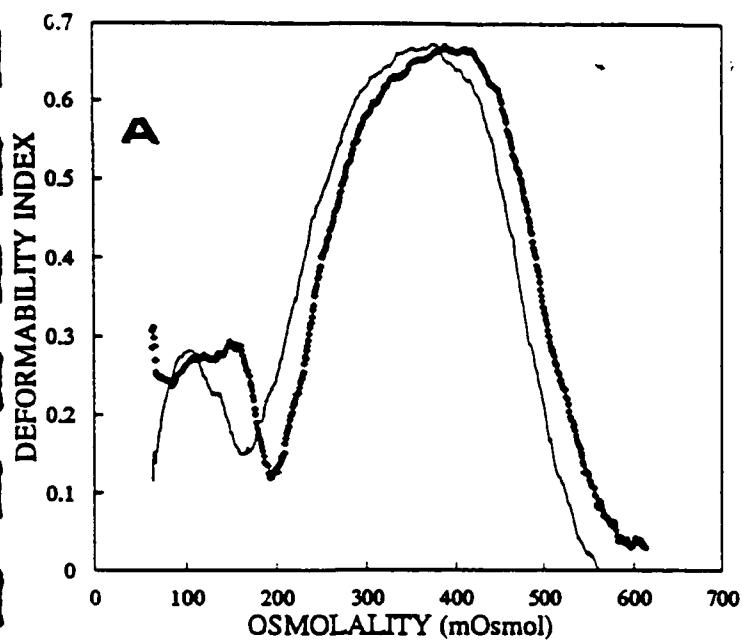


FIGURE 4: GAMMA IMAGE RESULTS FROM SUBJECT # 1. Note that A =Heart; B= Liver and C=Spleen.

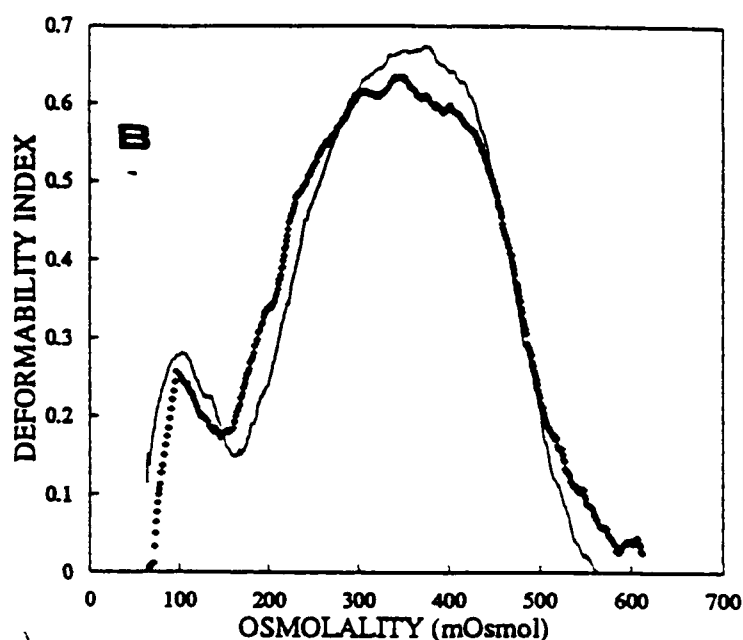


Figure 5: Comparison of osmotic fragility of reconstituted, lyophilized red blood cells and control RBC stored in CPDA for a comparable period at +4C. Osmotic fragility curves of freshly drawn RBC are included for comparison with the present data.

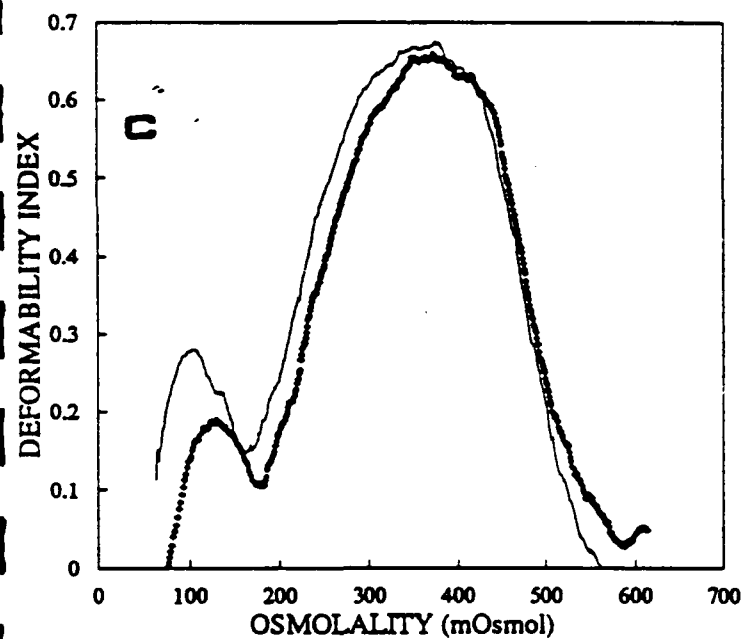




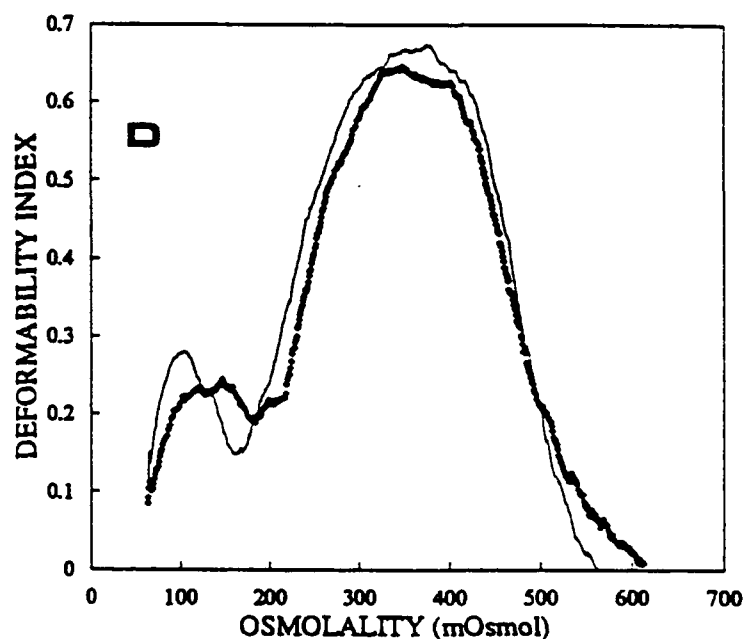
— Freshly Drawn RBC · Cont RBC stored in CPDA



— Freshly Drawn RBC · Lyo RBC stored in CPDA



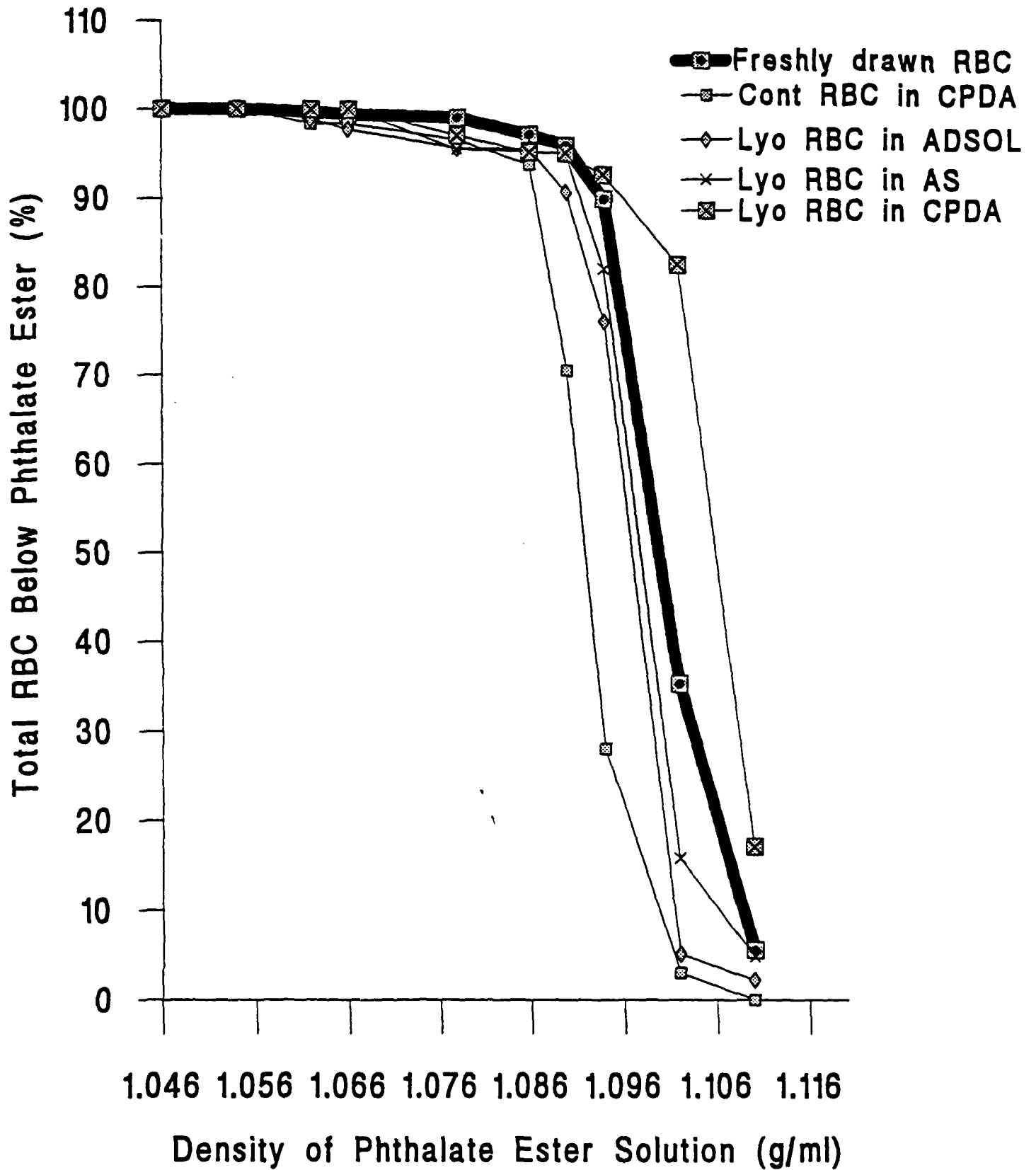
— Freshly Drawn RBC · Lyo RBC stored in ADSOL



— Freshly Drawn RBC · Lyo RBC stored in AS

Figures 6A-6D: Osmotic deformability profile for rehydrated, lyophilized RBC and control RBC stored in CPDA for a comparable period (age of cell = 16 days). Osmoscan of freshly drawn RBC is included for comparison with experimental sample. The osmotic deformability profiles indicate the responses of the RBC to different osmotic stresses at a fixed shear stress of 230 dynes/cm². It allows for a detailed analysis of the various factors that determine the rheological properties of RBC. The essential features of these curves are : 1) Hypotonic response of the RBC is determined primarily by the surface area to volume ratio. Cells with a high ratio show a left shift (i.e. increased osmotic resistance); 2) Hypertonic response is determined by internal viscosity of the RBC; 3) Membrane rigidity. Note that at physiological osmolality (300mOsmol) the deformability of RBC is maximal. Reduction in the DI_{max} is a reflection of either an increase in membrane rigidity or a reduction in surface area.

Figures 7: Density distribution profile of rehydrated, lyophilized RBC and control RBC stored in CPDA for 16 days at +4C.





DRAFT PROTOCOL

**A STUDY TO DETERMINE CLEARANCE OF ⁵¹Cr-LABELLED AUTOLOGOUS
ERYTHROCYTES IN CYNOMOLGUS MONKEYS FOLLOWING RED BLOOD CELL
LYOPHILIZATION, RECONSTITUTION AND LABELLING**

Study Number: 2-J35

Sponsor: Cryopharm Corporation
2585 Nina Street
Pasadena, California 91107

Telephone Number: (818) 793-1040
FAX Number: (818) 793-1445
Emergency Number: ()

**Sponsor's
Representative:** Raymond P. Goodrich, Ph.D.

Testing Facility: TSI Mason Laboratories
57 Union Street
Worcester, MA 01608

Telephone Number: (508) 791-0931
FAX Number: (508) 753-1834

Study Director: Paul A. Zavorskas, M.A.

Protocol Issue Date: February 12, 1992

1. OBJECTIVE: To determine the clearance of autologous erythrocytes labelled with ^{51}Cr in cynomolgus monkeys following red blood cell lyophilization, reconstitution and labelling.

2. PROPOSED STUDY TIMETABLE: Study dates will be addressed by amendment.

Initiation of Dosing:
Termination:
Report Date (Audited Draft):

3. EXPERIMENTAL DESIGN: The test articles will consist of two experimental samples of freeze-dried and reconstituted monkey erythrocytes, tagged *in vitro* with ^{51}Cr . Blood samples (50 ml) from the two monkeys will be collected in CPD-A buffer at a 1:8 ratio and shipped on ice via overnight delivery to the Sponsor. The two monkey blood samples will be freeze-dried by the Sponsor using its proprietary method. Freeze-dried cells and reconstitution reagents will be shipped by the Sponsor via overnight delivery to the Test Facility for reconstitution and labelling. The ^{51}Cr -labelling procedure is presented in Attachment 2.

The two monkeys will each receive a single administration of the autologous, lyophilized and reconstituted, ^{51}Cr -labelled packed erythrocytes via an intravenous catheter in a peripheral vein. Administration of packed erythrocytes and subsequent blood collections will be performed on animals tranquilized with ketamine HCl. Following administration of labelled packed red cells into each monkey, a peripheral blood sample (≈ 1.0 ml each) will be collected into heparinized tubes from a blood vessel other than the one used for administration of labelled RBCs at the timepoints listed below. The disposition of the monkeys will be determined by the Study Director in consultation with the Sponsor.

A. Study Design: Test article information is included in Attachment 1.

Group Number	Number of Males	Treatment	Dose Level	Route	Frequency/Duration	Blood Collection Timepoints
1	2	^{51}Cr -labelled packed erythrocytes (autologous)	50 ml	Intravenous	Once/Day 1	5, 7.5, 10, 15, 30, 60 min. 2, 4, 8, 12, 24, 48, 72 hr

4. ANIMALS: The study will consist of two non-naive monkeys. The monkeys were obtained from a source approved by the Test Facility. Specifics concerning the source of the monkeys and any previous treatments are documented in the Study File.

Species: Monkey; cynomolgus
Strain: Macaca fascicularis
Number of Males: 2
Age at Initiation of Dosing: Young adult
Minimum Weight at Initiation of Dosing: 7.0 kgs; the body weights of test animals at the initiation of dosing will be documented in the raw data.

A. Quarantine: The monkeys have undergone a period of quarantine. During this period, three intradermal tuberculin tests were conducted, at two week intervals, on each monkey. The monkeys were released from quarantine after the third consecutive negative tuberculin test. In addition, various samples were collected: blood for a complete blood cell count and serum chemistry profile, rectal swab for culture, and feces for occult blood, ova and parasite determinations. A physical examination, including an ophthalmic examination, was performed on each monkey. All observations and sample collections were recorded during the quarantine period, in accordance with the approved Standard Operating Procedures of the Test Facility.

B. Identification: Each animal will be identified by a unique number and this number will be posted on the animal's cage. All animals will be permanently identified by tattoo.

5. ANIMAL CARE PROCEDURES: The procedures discussed below will be performed in accordance with the Standard Operating Procedures of the Test Facility.

A. Animal Husbandry: Animal husbandry will be performed according to the approved Standard Operating Procedures of the Test Facility. Monkeys will be singly housed. During quarantine, husbandry supplies and schedules will be identical to those of the test period. A twelve hour light/dark cycle will be maintained.

B. Environmental Conditions: Environmental conditions in the animal quarters will be maintained according to the Standard Operating Procedures of the Test Facility (and as described in the Guide for the Care and Use of Laboratory Animals, NIH Publication No. 86-23, Revised 1985). Room temperature will be maintained between 18 - 26° C and the humidity range will be 40 - 70%.

C. Diet: Purina Monkey Chow[®] 5038 will be fed daily. Diet will be supplemented with fresh produce.

D. Water: Filtered (5 μ pore size) tap water will be provided to the animals *ad libitum*. A water analysis (latest annual analysis), conducted by an independent certified test facility, will be maintained in the study file. The analysis will include the following: heavy metals, chlorinated hydrocarbons, organophosphates, nitrates, nitrites, standard plate count, total trihalomethanes, and dissolved minerals.

E. Contaminants: The Study Director and/or Sponsor have considered possible interfering substances potentially present in the animal feed and water, including the test material itself or possible structurally-related materials as well. None of these contaminants are reasonably expected to be present in animal feed or water at levels sufficient to interfere with this study.

6. TECHNICAL PROCEDURES: The procedures discussed below will be performed in accordance with the Standard Operating Procedures of the Test Facility. Clinical observations will be recorded manually or on LABCAT[®]. All other technical procedures study data will be recorded manually.

A. Treatment: Animals will be treated with a single dose of autologous, lyophilized and reconstituted, ^{51}Cr -labelled packed erythrocytes via an intravenous catheter in a peripheral vein. Administration of the test article will be documented for each animal; this documentation will include the volume and time of the dose administration.

B. Clinical Observations: Cageside observations will be performed daily for mortality, moribundity, and indications of a toxic and/or pharmacologic effect. Data for specific observations will be recorded by exception, noting those animals for which a specific observation is made.

C. Body Weights: Individual body weights will be recorded on Day 1 prior to treatment, and at 72 hours post-treatment.

D. Blood Collection: Blood samples (≈ 1.0 ml/sample) will be collected into heparinized tubes from a blood vessel other than the one used for test article administration at 5, 7.5, 10, 15, 30 and 60 minutes, and 2, 4, 8, 12, 24, 48, and 72 hours post-administration. Samples will be processed and evaluated for ^{51}Cr activity. All counts will be corrected for natural decay of ^{51}Cr during the study period.

E. Moribund Animals: Animals with life-threatening clinical signs which indicate that they are unlikely to survive until the next scheduled observation will be euthanized. Euthanasia of moribund animals will be authorized by the Study Director or staff veterinarian.

7. LABORATORY PROCEDURES: The procedures discussed below will be performed in accordance with the Standard Operating Procedures of the Test Facility.

A. Blood Samples: Blood samples will be processed and evaluated for ^{51}Cr activity. When possible, duplicate 0.2 ml aliquots of each blood sample will be analyzed for ^{51}Cr activity in a Packard Model 5650 gamma scintillation counter.

8. PATHOLOGY PROCEDURES: The procedures discussed below will be performed in accordance with the Standard Operating Procedures of the Test Facility.

A. Euthanasia: Euthanasia (overdose of sodium pentobarbital), if required, will be performed in accordance with accepted American Veterinary Medical Association (AVMA) guidelines (Report of the American Veterinary Medical Association (AVMA) Panel on Euthanasia Journal of American Veterinary Medical Association, 188:252-268, 1986).

9. RECORDS TO BE MAINTAINED: In addition to routine equipment maintenance and/or calibration logs and quality control records, the following will be maintained.

Test Article:

- Test article receipt, storage, usage, and disposition records
- Shipping records
- Blood reconstitution records
- ^{51}Cr labelling records

Facility Records:

- Temperature and humidity records
- Water analysis
- Feed identification
- Cage and rack placement records

In-Life Phase:

- Requisition and receipt of animals
- Quarantine observation, health assessment and release records
- Dosing records
- Clinical observation records
- Body weight records
- Sample collection records

Sample Collection Records:

- Sample processing records
- Blood ^{51}Cr counting records

Other:

All other records that would be required to reconstruct the study and demonstrate adherence to the protocol.

10. DATA ANALYSIS: The total amount of ^{51}Cr activity administered with each dose will be calculated. Blood samples will be analyzed to determine ^{51}Cr levels. Blood ^{51}Cr levels vs. time data will be analyzed to determine the following parameters: area under the curve (AUC), volume of distribution (Vd), half-lives of distribution(s) and elimination phases, systemic or total body clearance (Cl_s) and mean residence time (MRT).

11. PROTOCOL CHANGES: If it becomes necessary to change the approved study protocol, verbal agreement to make this change will be made between the Study Director and the Sponsor. This change and the justification will be documented in writing and signed by both the Study Director and the Sponsor and attached to the protocol as an amendment.

12. STUDY DEVIATIONS: Should a deviation from the protocol or an SOP occur, the circumstances, the action taken, and the impact on the study will be assessed immediately by the Study Director and documented. The Sponsor will be notified by the Study Director as soon as is possible.

13. MISCELLANEOUS: If requested, copies of relevant Standard Operating Procedures will be made available to the Sponsor for inspection on-site. Copies of all raw data and the final report will be maintained in the Test Facility archives at 57 Union Street, Worcester MA and will be indexed by report number.

14. REPORTS:

A. Preliminary Report: Three copies of the preliminary report will be submitted within a time frame to be determined when the study is scheduled. The report will include:

- Name and address of Test Facility
- Study dates
- Test and control article identification, characterization, and stability
- Identification of test system
- Scientific personnel
- Experimental design and methods
- Treatment and disposition summary
- Clinical observation summary
- Body weights summary
- A tabulated summary of the results as they relate to the study objective
- The location where the raw data and specimens will be stored
- Deviations from the Test Facility's Standard Operating Procedures or the protocol
- Objectives and procedures stated in the original protocol, and any changes in the original protocol

B. Final Report: Three copies of the final report will be submitted within fourteen working days after resolution of items arising from the preliminary report.

ATTACHMENT 1

INFORMATION AND INSTRUCTIONS REQUIRED FROM SPONSOR

TEST ARTICLE INFORMATION:

1. Physical Data:

<u>Name/ Code</u>	<u>Physical Description Color/Form</u>	<u>Lot/Batch Number</u>	<u>Expiration Date</u>
-----------------------	--	-------------------------	----------------------------

Erythrocytes

Monkey # _____

Monkey # _____

2. **Characterization/Stability:** The characterization and stability of the freeze-dried erythrocytes has been or will be performed by the Sponsor.

3. **Manufacturer:**

4. **Supplier:** (if different from manufacturer)

5. **Bulk Analysis/Reanalysis:** responsibility of the Sponsor.

6. **Storage Condition (check appropriate):**

[] Protect from light
Store at [] Room Temperature; [] 2 - 8°C; [] ≤-15°C; [] ≤-70°C
[] Other (specify) _____

7. **Hazards (check one):**

[] None known, take standard precautions
[] Other (specify)

8. **Special Instructions (check one):**

[] None
[] Other (specify)

The Sponsor will be responsible for the performance and documentation of the freeze-drying process of the erythrocytes.

ATTACHMENT 1 (Continued)

INFORMATION AND INSTRUCTIONS REQUIRED FROM SPONSOR

RECONSTITUTION BUFFER:

1. Physical Data:

<u>Name/ Code</u>	<u>Physical Description Color/Form</u>	<u>Lot/Batch Number</u>	<u>Expiration Date</u>
-----------------------	--	-------------------------	----------------------------

2. Manufacturer:

3. Supplier: (if different from manufacturer)

4. Storage Condition (check as appropriate):

Store at Protect from light
 Room Temperature; 2 - 8°C; ≤-15°C; ≤-70°C
 Other (specify) _____

WASH BUFFER:

1. Physical Data:

<u>Name/ Code</u>	<u>Physical Description Color/Form</u>	<u>Lot/Batch Number</u>	<u>Expiration Date</u>
-----------------------	--	-------------------------	----------------------------

2. Manufacturer:

3. Supplier: (if different from manufacturer)

4. Storage Condition (check as appropriate):

Store at Protect from light
 Room Temperature; 2 - 8°C; ≤-15°C; ≤-70°C
 Other (specify) _____

ATTACHMENT 1 (Continued)

INFORMATION AND INSTRUCTIONS REQUIRED FROM SPONSOR

FORMULATION INSTRUCTIONS (check as appropriate):

- The reconstitution and labelling of the test articles will be performed by the Sponsor off-site.
- The reconstitution and labelling of the test articles will be performed by the Sponsor on-site.
- Instructions for reconstitution and labelling of the test articles will be provided by the Sponsor for performance by Test Facility personnel.

Reconstituted samples will be stored according to these instructions:

- Discard after one use
- Protect from light
- Store at Room Temperature; 2 - 8°C; ≤-15°C; ≤-70°C
- Other (specify) _____

ARCHIVING INSTRUCTIONS

All records and raw data will be inventoried and handled as follows: (check one):

- stored by the Test Facility after submission of the final report.
- transferred to the Sponsor after the submission of the final report.

ATTACHMENT 2

⁵¹Cr LABELLING PROCEDURE

The reconstituted cells will be tagged with ⁵¹Cr using the sodium chromate technique. It is expected that each 0.1 ml of packed red cells will require 5 microcuries of ⁵¹Cr sodium chromate.

The *in vitro* labelling procedure is as follows:

This protocol is modified from a procedure described by J.C. Sisson in the CRC Manual of Nuclear Medicine Procedures, p. 134.

- 1) The sample of erythrocytes in p-PBS buffer will be placed at room temperature (RT).
- 2) To the sample add ⁵¹Cr sodium chromate available from Amersham as a 1 mCi/ml sterile sodium chloride solution. Add 5 microcuries of ⁵¹Cr for every 0.1 ml of packed RBC pellet.
- 3) Incubate the sample at RT for 30 minutes. Stop the labelling reaction by adding 2 microliters of ascorbic acid (50 mg/ml in PBS) to every 0.1 ml of pellet. The pellet is then allowed to sit another 2 minutes at RT.
- 4) The sample will be washed 2 to 3 times using the Sponsor's sterile wash buffers (to be provided by the Sponsor) and gentle centrifugation, to remove unincorporated isotope.
- 5) Washed packed erythrocytes are now ready for immediate reinfusion into each test animal.

ACCEPTANCE OF PROTOCOL

A STUDY TO DETERMINE CLEARANCE OF ^{51}Cr -LABELLED AUTOLOGOUS ERYTHROCYTES IN CYNOMOLGUS MONKEYS FOLLOWING RED BLOOD CELL LYOPHILIZATION, RECONSTITUTION AND LABELLING

Study Number: 2-J35

Sponsor: Cryopharm Corporation
2585 Nina Street
Pasadena, California 91107

Signature/Date
Raymond P. Goodrich, Ph.D.
Director, Research & Development

Test Facility: TSI Mason Laboratories
57 Union Street
Worcester, MA 01608

Signature/Date
Paul A. Zavorskas, M.A.
Study Director

THIS STUDY DOES NOT UNNECESSARILY DUPLICATE ANY PREVIOUS EXPERIMENTS. TO THE BEST OF
OUR KNOWLEDGE, THERE ARE NO ALTERNATIVES TO ANIMAL USE FOR THIS TYPE OF STUDY.

CRYOPHARM CORPORATION

PROTOCOL

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

April 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 six 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 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.

4.0

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:

<u>Height Without Shoes (cm)</u>	<u>Weight Indoor Clothing Without Shoes (kg)</u>
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 -12)

The following will be obtained twelve 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 whole blood 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_sA_g) 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). Two aliquots (3-5 ml each) of the reconstituted red blood cells will be used to inoculate blood culture bottles. A sample will also be sent to the blood bank for type and crossmatch.
- 5.4.2 **Chromium Labeling of Red Blood Cells:** The re-hydrated blood will be brought to the Nuclear Medicine Department where a 30-35 ml aliquot will be removed from the Cobe bag using sterile techniques. These red cells will be incubated for 15 minutes at 37°C 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 needle into the contralateral arm.
- 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>Time</u>	<u>Post-transfusion Blood Collection Times</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 (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

INFORMED CONSENT

NOTE: Appendix I to Protocol

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 ⁵¹chromium 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.

Signature of Volunteer

Date

Clinical Coordinator

Date

APPENDIX II

The Lyophilization of Red Blood Cells at Cryopharm

Each unit of whole blood 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 whole blood 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°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°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 one wash with isolation buffer for two minutes, and two more washes with a Buffered Dextrose Saline-based solution for five minutes at each wash. The total processing time must be less than two hours. 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. 30 to 35 ml of washed, reconstituted lyophilized erythrocytes (RBC) are added to an empty sterile vial. Use a sterile air vent to vent the vial before injecting the red blood cells.
2. 5 ml of RBC are injected into a purple top tube to be used as the background control.
3. ⁵¹Chromium (150 to 200 microcuries) are added to the vial which is gently swirled to maintain the RBC in suspension.
4. Incubate at 37°C for 15 minutes; swirl the vial gently on occasion.
5. Add ascorbic acid (100 mg) to the vial.
6. Draw 5 ml of labeled RBC into a hypodermic syringe, and inject into a purple top tube for standard control.
7. Draw 20 to 25 ml of labeled RBC into a fresh hypodermic syringe.
8. Weigh the filled syringe and empty 18-19 gauge butterfly set.

9. 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.
10. Weigh empty syringe and butterfly set after injection of blood.
11. 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 ⁵¹Cr 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, the mean corrected cpm of the duplicate 5, 7.5, 12, and 15 minute samples are plotted versus time, and an estimated time zero is obtained from the y intercept of a best straight line through the points.

7. Calculate percent recovery of label for each sample as $R = (T/S) \times 100$ where $R = \% \text{ recovery}$, $S = \text{extrapolated Time Zero CPM}$, $T = \text{corrected timed sample mean CPM}$.
8. Determine apparent $T_{1/2}$ of ^{51}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. Make 1:10 and 1:100 dilutions of patient STANDARD in 0.9% saline.
2. Pipette 1 ml of the diluted specimens and of the 5 minute and 15 minute samples into each of two 5 ml test tubes.
3. Cap and mix test tubes. Count for 30 minutes in a gamma counter.
4. Total Injected Dose (cpm) = $(A \times B \times C)$

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

5. Theoretical Time Zero = $\frac{\text{Total Injected Dose}}{\text{Whole Blood Volume}}$

Where whole blood volume is estimated as shown below.

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

Preservation of metabolic activity in lyophilized human erythrocytes

(lyophilization/pentose phosphate shunt/methemoglobin/glycolytic enzymes)

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Preservation of metabolic activity in lyophilized human erythrocytes

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Communicated by John D. Baldeschweiler, October 10, 1991

ABSTRACT Normal human erythrocytes (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, we studied the effects of our lyophilization process on RBC metabolism. Of all the metabolic enzymes studied, only triosephosphate isomerase (D-glyceraldehyde-3-phosphate ketol-isomerase, EC 5.3.1.1), enolase (2-phospho-D-glyceratehydro-lyase, EC 4.2.1.11), and pyruvate kinase (ATP:pyruvate *O*²-phosphotransferase, EC 2.7.1.40) were decreased when compared with fresh control nonlyophilized RBC. The activities of these enzymes did not differ significantly from those of blood bank RBC. Concentrations of high-energy intermediates, ATP, and 2,3-diphosphoglycerate, along with lactate and ATP production were decreased in lyophilized RBC. No enzymes of the pentose phosphate shunt were altered during lyophilization. In addition, our data show that lyophilized RBC possess an intact capacity to (i) synthesize adenine nucleotides and (ii) reduce MetHb to Hb and, thus, maintain the Hb in a functional physiologic state similar to fresh nonlyophilized RBC. The present study demonstrates the possibility of lyophilizing RBC in a manner that maintains normal metabolic and enzymatic function upon rehydration.

Maintenance of the metabolic functions of human erythrocytes (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 allows for a 35- to 42-day shelf life, or frozen storage at -80°C, which permits long-term storage (up to 10 yr) but requires maintenance of low temperature (3, 4). The ability to freeze-dry RBC would eliminate this need for low storage temperatures. A successful process would require that damage to the cells normally induced by drying be avoided. Cells treated in this manner must retain normal cytoskeletal, metabolic, and oxygen-transporting functions. In this paper we examined the effects of freeze-drying on one of these characteristics—namely, metabolic function. Several previous studies have shown promise in this regard by demonstrating that certain protectants are effective in preserving the enzyme activities and functions of dry biological specimens (5). Until this time, however, no comparable demonstration has been provided due to limitations of available methods for protecting cells from damage during drying.

Normal adult human RBC generate energy almost exclusively through the metabolism of glucose primarily via the Embden-Meyerhof pathway and the pentose phosphate shunt (PPS) (Fig. 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, ATP and 2,3-diphosphoglycerate (2,3-DPG) act to regulate the oxygen affinity of Hb. The above glycolytic pathway requires proper functioning of the various glycolytic enzymes for the formation of the intermediates essential for the RBC to transport oxygen and to maintain those physical characteristics required for its *in vivo* survival in circulation. Abnormal metabolic activities have been seen in RBC 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.

Because maintenance of proper metabolic functions of RBC is so important to carrying out RBC physiologic functions, this study was designed to evaluate preservation of the key RBC glycolytic enzyme activities after lyophilization and rehydration of RBC. Although maintenance of such features does not guarantee *in vivo* survival, salient data from our studies show that metabolic functions of rehydrated lyophilized RBC stored at 22°C are comparable to that of blood bank RBC stored for equivalent periods in a hydrated state at 4°C.

MATERIALS AND METHODS

After informed consent was obtained, blood was drawn from six healthy adult individuals having 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 anticoagulant by using conventional blood banking techniques. The blood units (500 ml each) were centrifuged at 1800 × *g* for 5 min 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), with an automated cell washer (model 2991, Cobe, Lakewood, CO). The cells were then lyophilized, according to procedures that have been described in detail (12). Samples prepared in this manner consistently exhibit moisture contents of 1-3%, as measured by the Karl Fischer method (13). At day 10, the dried RBC were rehydrated and reconstituted in phosphate-buffered rehydration buffers (360 mosmol, pH 7.4, Cryopharm, Pasadena, CA) at 22°C. Note, however, that with our current procedures 7 days is the time required to reduce the residual moisture content of the sample to a level (1-2%) that allows storage at ambient temperatures. Briefly, to rehydrate the RBC, 600 g of rehydration buffer was added to the dried RBC and then agitated on a wrist action shaker (Burrell, Pittsburgh) until the RBC were fully rehydrated (usually for 15 min). At the end of the rehydration step, the

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Abbreviations: TPI, triose phosphate isomerase; PK, pyruvate kinase; 2,3-DPG, 2,3-diphosphoglycerate; PPS, pentose phosphate shunt; RBC, erythrocyte(s).

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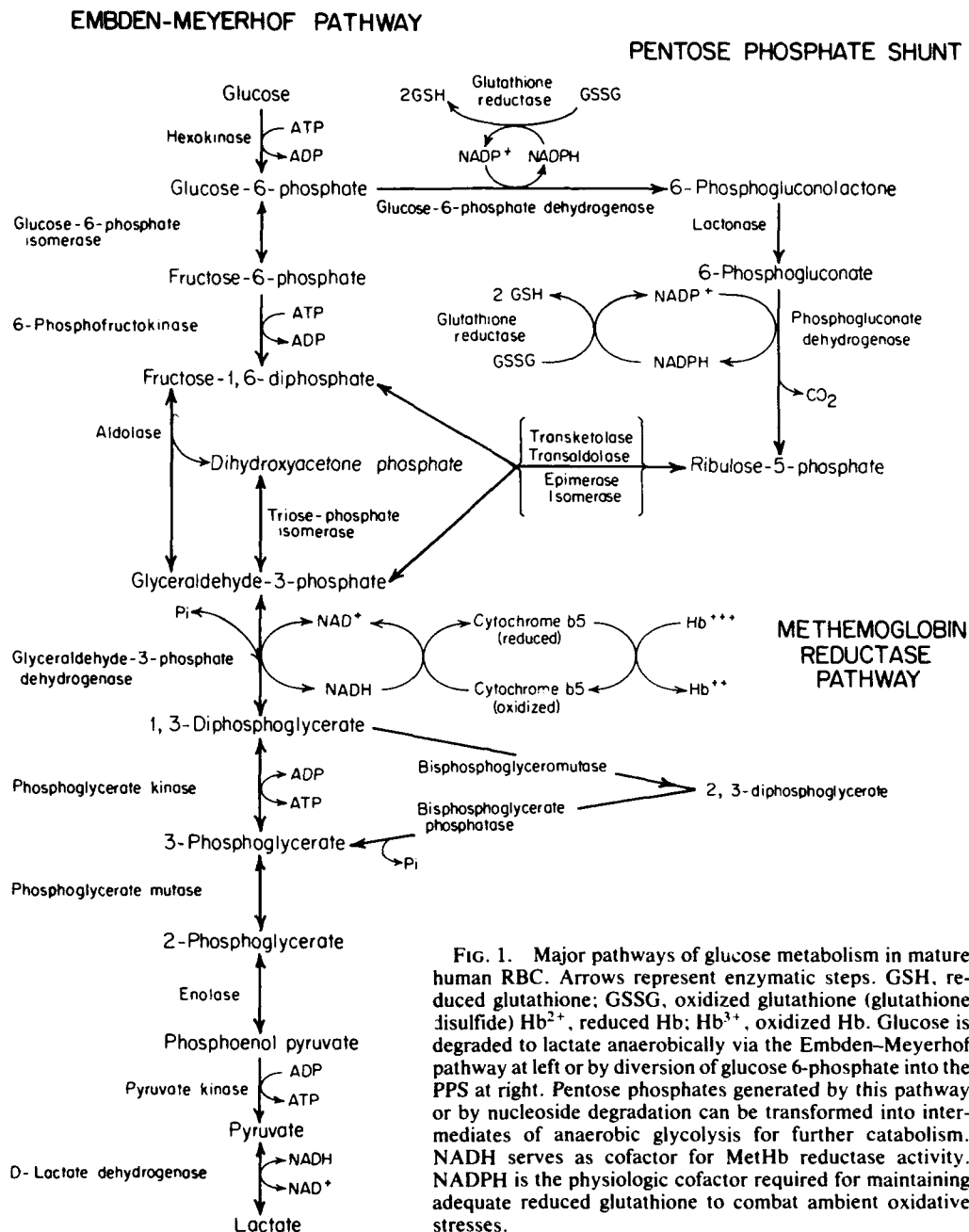


FIG. 1. Major pathways of glucose metabolism in mature human RBC. Arrows represent enzymatic steps. GSH, reduced glutathione; GSSG, oxidized glutathione (glutathione disulfide); Hb^{2+} , reduced Hb; Hb^{3+} , oxidized Hb. Glucose is degraded to lactate anaerobically via the Embden-Meyerhof pathway at left or by diversion of glucose 6-phosphate into the PPS at right. Pentose phosphates generated by this pathway or by nucleoside degradation can be transformed into intermediates of anaerobic glycolysis for further catabolism. NADH serves as cofactor for MethHb reductase activity. NADPH is the physiologic cofactor required for maintaining adequate reduced glutathione to combat ambient oxidative stresses.

RBC suspension was centrifuged at $1800 \times g$ for 10 min at $22^\circ C$. The supernatant was removed, and the packed RBC were washed twice in special isotonic wash buffers (12) by centrifugation at $1800 \times g$, using a Cobe automatic cell washer. Reconstituted RBC were assayed for glycolytic enzyme activities and intermediates, according to 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}C]adenine into the adenine nucleotide pool in intact RBC with the method of Zerez *et al.* (20). Because all RBC samples had endogenous glucose and exhibited low rates of [^{14}C]adenine incorporation without exogenous glucose, a control was included in which incorporation was measured with 1.0 mM iodoacetate, an inhibitor of glycolysis. This step allowed the assessment of "background" [^{14}C]ad-

enine incorporation (i.e., under conditions of total inhibition of glycolysis) by subtracting [^{14}C]adenine incorporation in the presence of iodoacetate from incorporation in its absence. Thus, reported rates of [^{14}C]adenine incorporation were corrected for this background incorporation.

Rate of MethHb Reduction. The rate of MethHb reduction in intact RBC was determined by using a 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 nonlyophilized RBC were analyzed with a two-tailed Student's *t* test for paired data. Comparisons between lyophilized and blood bank-stored RBC were made by using the two-tailed Student's *t* test for independent data.

RESULTS

Activities of Glycolytic Enzymes. Results of the measurements of glycolytic enzyme activities for hemolysates from rehydrated lyophilized RBC and nonlyophilized fresh RBC

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

Enzyme	Enzyme activity, $\mu\text{mol}/\text{min}\cdot\text{g}$ of Hb				
	Lyo	N-lyo	B-Bank	N-R	P
HX	1.26 \pm 0.2	1.65 \pm 0.1	1.20 \pm 0.1	0.98–1.3	NS
PGI	44.7 \pm 4.6	44.3 \pm 2.7	48.3 \pm 6.0	43.7–65.8	NS
PFK	12.1 \pm 1.6	11.7 \pm 1.0	9.73 \pm 2.2	8.44–12.2	NS
Ald	3.59 \pm 0.4	3.72 \pm 0.5	2.39 \pm 0.3	1.97–3.59	NS
TPI	1750 \pm 460	2140 \pm 490	2900 \pm 777	2130–3340	<0.005
G3PD	318 \pm 68	311 \pm 43	244 \pm 72	238–346	NS
DPGM	5.34 \pm 0.7	4.64 \pm 0.9	8.43 \pm 2.2	3.93–5.9	<0.015
PGK	340 \pm 147	340 \pm 115	349 \pm 47.7	212–341	NS
PGM	35.2 \pm 5.1	38.1 \pm 6.0	17.3 \pm 6.7	13.9–38.0	NS
Eno	4.99 \pm 1.0	7.60 \pm 0.9	4.96 \pm 0.9	4.2–6.6	<0.001
PK	18.9 \pm 5.7	21.1 \pm 5.4	15.0 \pm 2.1	12.5–17.2	<0.032
LDH	231 \pm 29	190 \pm 19	141 \pm 56	145–203	<0.001
G6PD*	12.4 \pm 1.6	14.7 \pm 1.8	ND	9.90–13.2	NS
6PGD*	11.1 \pm 1.0	10.0 \pm 1.1	ND	7.27–10.0	NS
TA*	0.97 \pm 0.2	1.10 \pm 0.3	ND	0.78–1.3	NS
TK*	0.68 \pm 0.1	0.93 \pm 0.7	ND	0.50–1.0	NS

Data represent the mean \pm SD for six 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 10 days before analysis. Lyo, lyophilized, 10 days old; N-lyo, nonlyophilized freshly drawn blood; B-Bank, blood bank; N-R, normal range for freshly drawn blood; ND, not detected; NS, not significant (comparisons were made between lyophilized and nonlyophilized 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); G3PD, glyceraldehyde-3-phosphate dehydrogenase (D-glyceraldehyde-3-phosphate:NAD⁺ 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-glycerate hydro-lyase, EC 4.2.1.11); LDH, D-lactate dehydrogenase (lactate:NAD⁺ oxidoreductase, EC 1.1.1.28); G6PD, glucose-6-phosphate dehydrogenase (D-glucose-6-phosphate:NADP⁺ 1-oxidoreductase, EC 1.1.1.49); 6PGD, phosphogluconate dehydrogenase (6-phospho-D-gluconate:NAD(P)⁺ 2-oxidoreductase, EC 1.1.1.43); TA, transaldolase (sedoheptulose-7-phosphate:D-glyceraldehyde-3-phosphate glyceronetransferase, EC 2.2.1.2), TK, transketolase (sedoheptulose-7-phosphate:D-glyceraldehyde-3-phosphate glycolaldehydetransferase, EC 2.2.1.1).

*Enzymes of PPS.

from autologous donors are summarized in Table 1. The range of enzyme activities for normal RBC and citrate phosphate dextrose-adenine-stored RBC are compared in Table 1. The activity of hexokinase in hemolysates was the same in rehydrated lyophilized RBC and nonlyophilized control RBC and was also similar to citrate phosphate dextrose-adenine-stored RBC. The activities of both triose-phosphate isomerase (TPI; D-glyceraldehyde-3-phosphate ketol-isomerase, EC 5.3.1.1) and pyruvate kinase (PK; ATP: pyruvate O²-phosphotransferase, EC 2.7.1.40, EC 2.7.1.40) were reduced in hemolysates from lyophilized RBC but were not different from blood bank-stored RBC (Table 1). Hemolysates from lyophilized RBC had TPI activity of 1750 $\mu\text{mol}/\text{min}\cdot\text{g}$ of Hb, which is significantly lower than that of control nonlyophilized RBC, 2140 $\mu\text{mol}/\text{min}\cdot\text{g}$ of 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 because it is present in excess in RBC. The activity of PK in hemolysates of rehydrated lyophilized RBC was 18.9 $\mu\text{mol}/\text{min}\cdot\text{g}$ of Hb compared with 21.1 $\mu\text{mol}/\text{min}\cdot\text{g}$ of Hb in nonlyophilized control RBC, $P < 0.03$. The activity of PK in blood bank RBC was 15.0 \pm 2.14 $\mu\text{mol}/\text{min}\cdot\text{g}$ of Hb, and this value is lower than that of lyophilized RBC. Because 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 D-lactate hydrogenase and bisphosphoglycerate mutase are significantly higher in lyophilized RBC than in fresh control nonlyophilized RBC, $P < 0.001$ and

$P < 0.01$, respectively (Table 1). The activities of the remaining Embden–Meyerhof pathway enzymes other than the above are similar in lyophilized, nonlyophilized, and blood bank RBC (Table 1).

Levels of Glycolytic Intermediates. The levels of glycolytic intermediates in intact rehydrated lyophilized and control nonlyophilized RBC are compared and summarized in Table 2. Significantly higher concentrations of dihydroxyacetate phosphate, 3-phosphoglycerate, 2-phosphoglycerate, phosphoenol pyruvate, ADP, and AMP were found in intact lyophilized RBC compared with nonlyophilized control (Table 2). On the contrary, the concentrations of ATP and 2,3-DPG are lower than in control RBC. Note that the concentrations of intermediates for normal RBC in Table 2 are much lower than those concentrations for either lyophilized or nonlyophilized RBC. These values were from fresh blood drawn directly into perchloric acid and then processed immediately for glycolytic intermediates. In contrast, lyophilized and nonlyophilized control RBC were processed before the isolation of perchloric acid extracts for intermediates and, therefore, would be expected to differ from normal values.

The rate of lactate production expressed as $\mu\text{mol}/\text{g}$ of Hb per hr is significantly reduced in lyophilized RBC, 6.60 \pm 3.58 compared with 10.6 \pm 2.86 in nonlyophilized control RBC, $P < 0.001$. Similarly, the rate of production of ATP by lyophilized RBC (0.368 \pm 0.173 $\mu\text{mol}/\text{g}$ of Hb per 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 six for both lyophilized and nonlyophilized RBC.

Table 2. Comparison of levels of glycolytic intermediates in rehydrated lyophilized and fresh nonlyophilized RBC

Intermediate	Glycolytic intermediate, nmol/g of Hb			P
	Lyo	N-lyo	NV	
G6P	49.8 ± 72.1	76.5 ± 102	100 ± 28.0	NS
F6P	0.92 ± 2.3	3.05 ± 7.5	15.6 ± 6.3	NS
FDP	760 ± 425	149 ± 179	4.70 ± 1.60	NS
DHAP	1770 ± 687	174 ± 147	37.5 ± 3.10	<0.012
GAP	112 ± 46.8	44.9 ± 43.5	9.38 ± 6.30	NS
2,3-DPG	3152 ± 938	9633 ± 2640	13,500 ± 2000	<0.004
3PG	611 ± 210	134 ± 56.1	122 ± 28.0	<0.006
2PG	338 ± 252	216 ± 165	31.3 ± 13.0	<0.046
PEP	216 ± 104	67.5 ± 50.8	50.0 ± 16.0	<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	<0.008
ADP	1743 ± 316	700 ± 133	409 ± 56.0	<0.003
AMP	2370 ± 343	204 ± 125	134 ± 25.0	<0.001

Data represent mean ± SD for six samples. Normal values are included for comparison with present data. Lyo, lyophilized, 10 days old; N-lyo, nonlyophilized freshly drawn blood; NV, normal values for freshly drawn blood; P, probability for comparisons between lyophilized and nonlyophilized RBC; G6P, glucose 6-phosphate; F6P, fructose 6-phosphate; FDP, fructose 1,6-disphosphate; DHAP, dihydroxyacetone phosphate; GAP, glyceraldehyde 3-phosphate; 3PG, 3-phosphoglycerate; 2PG, 2-phosphoglycerate; PEP, phosphoenol pyruvate; Pyr, pyruvate; Lact, lactate.

MetHb Reduction and Level of Reduced Glutathione. The semilogarithmic plots of the residual MetHb and the incubation time are shown in Fig. 2. MetHb reduction in rehydrated lyophilized and in nonlyophilized RBC followed similar exponential decay (Fig. 2). The rates of MetHb reduction, expressed as the half-life for all samples tested, are shown in Fig. 3. The mean rate of MetHb reduction in lyophilized RBC was 17.9 ± 4.30 hr and was not significantly different from control nonlyophilized RBC, 17.4 ± 4.80 hr, $P > 0.05$ (Fig. 3). The concentration of reduced glutathione in lyophilized RBC was 689 ± 136 $\mu\text{g/g}$ of Hb, which is significantly lower than that of nonlyophilized fresh RBC, 2150 ± 406 $\mu\text{g/g}$ of Hb, $P < 0.001$. Although the concentration of reduced glutathione was lower 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 nonlyophilized RBC = 6.44 $\mu\text{mol/min}\cdot\text{g}$ of Hb] and glutathione peroxidase (glutathione:hydrogen-peroxide oxidoreductase, EC 1.11.1.9; lyophilized RBC = 19.9 and nonlyophilized RBC = 21.6 $\mu\text{mol/min}\cdot\text{g}$ of Hb) were not different from control RBC.

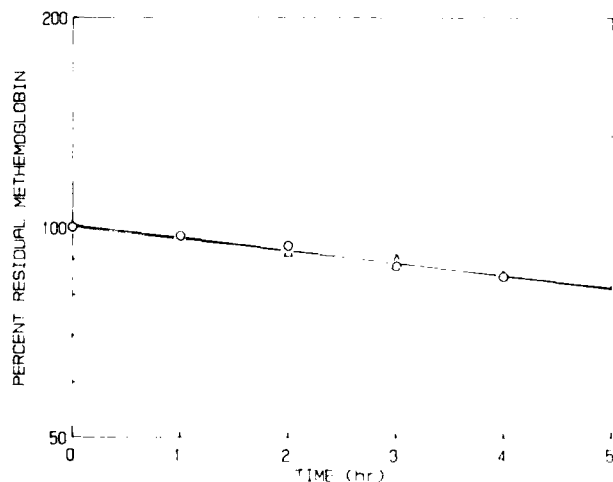


FIG. 2. Linear regression fit for semilogarithmic plots of percent residual MetHb vs. time of incubation for intact RBC from lyophilized (Δ) and nonlyophilized RBC (\circ). Data are means of six samples.

Rate of [^{14}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}C]adenine into adenine nucleotides. Nonlyophilized RBC had a rate of [^{14}C]adenine incorporation of 2.81 ± 0.40 nmol/min·ml of RBC (mean ± 1 SD, $n = 5$). In contrast, lyophilized RBC had a rate of incorporation of 0.407 ± 0.122 nmol/min·ml of RBC (mean ± 1 SD, $n = 5$), which decreased significantly ($P < 0.00005$) from nonlyophilized RBC. Although lyophilized RBC had a lower rate of [^{14}C]adenine nucleotide incorporation, the ability of these cells to incorporate adenine into nucleotides is noteworthy because only freshly obtained RBC have this function.

DISCUSSION

A major problem in blood preservation concerns the metabolic and functional lesions occurring during long-term storage of RBC in conventional liquid media. Storage lesions ultimately result in shortened RBC survival after transfusion (23). For satisfactory preservation of RBC, it is, therefore, important that the metabolic functions of the cells are well preserved. Frozen storage of RBC reduces this problem but

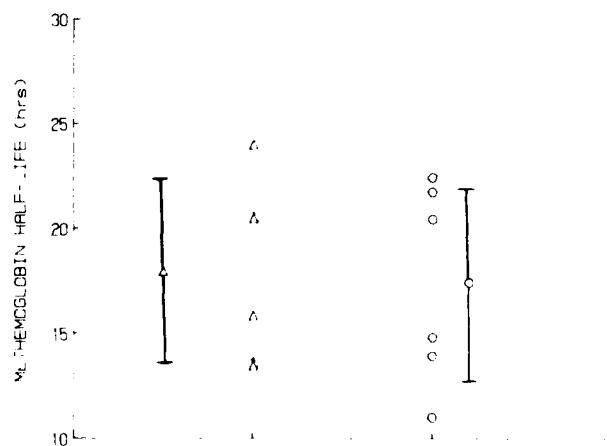


FIG. 3. Rate of MetHb reduction (expressed as half-life) in intact lyophilized (Δ) and nonlyophilized RBC (\circ). Mean ± SD MetHb half-life for both cell types are also shown.

requires the maintenance of low temperatures during storage and transport. Storage in the dry state requires that procedures be developed that protect the cells against dehydration-induced damage to enzymes, proteins, and membrane structure. The advantage of lyophilized RBC rests in the ability to store them for long periods under storage conditions that are far easier (i.e., room temperature, 22°C), making transport to sites of immediate need more feasible.

The relationship between glycolytic enzyme activities and freeze-dry processing of RBC has not been previously studied due to inabilities in producing intact and functional cells after drying. We have demonstrated that the activities of most enzymes involved in RBC metabolism are very well preserved in rehydrated lyophilized RBC prepared according to our procedures (12). Our data showed that of all enzymes studied, only TPI, enolase, and PK significantly decrease in lyophilized RBC when compared with fresh control nonlyophilized RBC. However, the activities of these enzymes do not significantly differ from those enzyme activities found in blood bank-stored RBC. TPI activity is the highest of any of the glycolytic enzymes by one or two orders of magnitude and may not be important in controlling glycolysis. That all major rate-limiting enzyme activities do not differ from these activities in fresh samples strongly suggests that the glycolytic pathway is functional in lyophilized RBC. It is interesting to also note that the activities of 6-phosphofructokinase and glucose-6-phosphate dehydrogenase have been reduced in RBC stored in conventional liquid media for an extended period (24). The impact of such changes in activities of 6-phosphofructokinase and glucose-6-phosphate dehydrogenase on the glycolytic pathway and posttransfusion 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. Because the major function of PPS is to produce NADPH and reduced glutathione, 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 MetHb reduction pathway is another important component of RBC metabolism. Decreased activity of this pathway may lead to accumulation of MetHb and 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 MetHb to functional Hb. The maintenance of both MetHb reduction and intact PPS suggests that lyophilized cells are not as likely to undergo Hb oxidation. Decreased antioxidant defenses in citrate phosphate dextrose-adenine-stored blood has been implicated as the mechanism responsible for the rapid removal of transfused RBC (26). That these defenses are still maintained in rehydrated lyophilized RBC further shows the efficiency of lyophilization technology in preserving the vital functions of RBC. Note also that PPS is important in the production of 5-phosphoribosylpyrophosphate, which is used by the RBC for synthesizing adenine nucleotides. These data also show that lyophilized RBC with their intact PPS can use an exogenous adenine source in the synthesis of adenine nucleotides.

Although concentrations of certain key glycolytic intermediates (ATP; 2,3-DPG) decrease in lyophilized cells, sufficient quantities of these intermediates remain for viability

and continuation of the various ATP- and 2,3-DPG-dependent functions as before lyophilization. In any event, the concentrations of ATP and 2,3-DPG in lyophilized cells resemble those of RBC in blood banking during their current storage life.

These data show that a freeze-drying process can be done so that the RBC maintain their metabolic functions upon rehydration. The results indicate a successful step in the direction of a lyophilized RBC product for transfusion medicine.

We thank Ms. Susanne M. Savely and Ms. Nemone Schulz for expert technical assistance. We also acknowledge the help of Mrs. Sharon Crandall, Mr. Jon Olson, and the various blood donors for this study. This project was supported, in part, by a Basic Research Grant N00014-90-C-0053, from the United States Naval Medical Research and Development Command.

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