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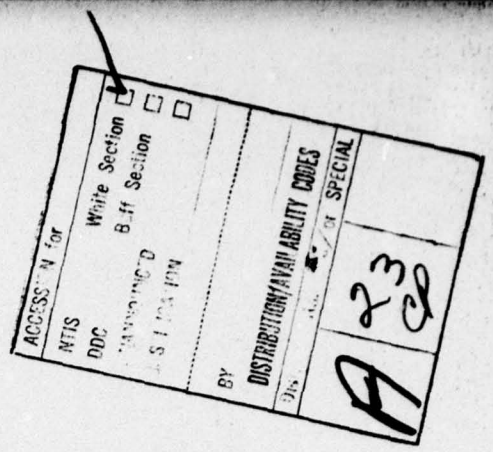

1. INTRODUCTION

1.1 General Background

The standard red cell preservatives, citrate-phosphate-dextrose (CPD) and acid-citrate-dextrose (ACD) permit red cells to be stored for up to 21 days. This period can be prolonged for approximately 2 more weeks by the addition of adenine, but adenine has not yet been licensed for use within the United States. Moreover, CPD and ACD result in rapid loss of the organic phosphate ester, 2,3-diphosphoglyceric acid (2,3-DPG) from the erythrocytes. Addition of adenine, while increasing the length of time that red cells may be stored without losing viability, does not improve the stability of 2,3-DPG. Indeed, it slightly accelerates the loss of this compound (1).

2,3-DPG is of potential importance in the storage of blood, since it exerts a profound effect upon the oxygen dissociation curve of red blood cells (2,3). Red cells that are depleted of 2,3-DPG have an increased oxygen affinity. When they are transfused, their ability to deliver oxygen is initially somewhat impaired, i.e., a lower tissue oxygen tension is required to extract oxygen from them. However, 2,3-DPG is relatively rapidly

replenished after cells are reinfused, one-half normal levels being achieved within about 4 hours, and regeneration being virtually complete at the end of 24 hours (4,5). Although it seems reasonable to suppose that 2,3-DPG is of physiologic importance, it has been very difficult to demonstrate that transfusion of 2,3-DPG depleted cells produces a deleterious effect. Recent investigations provide suggestive evidence that red cells with elevated 2,3-DPG levels improve cardiac function in Baboons in shock, when compared with 2,3-DPG depleted cells (6,7). Furthermore, studies in man suggest that red cells with normal 2,3-DPG may support cardiac output following cardiac bypass surgery in a manner which is superior to that of 2,3-DPG depleted cells. While the results of these studies are by no means compelling, it would seem desirable to infuse blood with normal 2,3-DPG content.

The use of "frozen blood" has received a great deal of attention in recent years. Claims have been made that red [redacted] stored at sub-freezing temperatures are a superior product, conferring lower risk of hepatitis and of leukocyte sensitization. If these claims are verified, then they do represent real advantages. But it should be emphasized that, as has been pointed out by a National Research Council Committee (8,9), the superiority of sub-zero stored red cells in these respects remains to be demonstrated. With the technology now available, and in the foreseeable future, frozen storage of red cells is prohibitively expensive, and,

as pointed out below, frozen storage is quite unsuitable for use under military field conditions.

An additional problem which requires consideration is that of microaggregates. A considerable literature is extant regarding the possible deleterious effect of these particles, which form rapidly when blood is stored in the liquid form (10,11,12,13,14,15). Microaggregates are believed to be comprised largely of platelets, leukocytes and particles of fibrin, and they may acutely increase the pulmonary artery pressure as they lodge in the lung (16,17, 18). A number of filters have been introduced to remove such microaggregates from stored blood, but some of these have been shown to be ineffective, and all are quite expensive (19,20). Moreover, these filters markedly limit the rate at which blood can be administered.

1.2 Military significance. The decrease in mortality among wounded soldiers during recent wars has, in large measure, been due to the large-scale utilization of blood and blood products in the severely injured. The heavy and complex equipment required for the freezing and subsequent deglycerolizing of frozen red cells makes frozen storage quite unsuitable for military field purposes, although it may play a role at major military medical installations.

Because of the unpredictable fluctuations in utilization of blood in the military situation, outdating of liquid stored blood is necessarily an even greater problem in the military than in civilian practice. Since large needs for

blood might arise at almost any time, sizeable inventories of blood must be kept at hand, and with a 3 week dating period, much of this blood will be wasted. With a 35 day or 42 day dating period, wasteage of blood would be less, and logistic problems attendant to collection and delivery of blood would be diminished.

If, in point of fact, 2,3-DPG levels are important in transfusions, they would be particularly so under military circumstances. Here, extensive wounding may result in replacement of the red cell mass several times within a few hours. These critically ill patients are the ones in whom a relatively small change in tissue oxygen tensions might make the difference between death and recovery. Micro-aggregates are believed to be of greatest hazard when large volumes of blood are transfused to individuals who already have impaired cardio pulmonary function. Wounded soldiers are precisely in this category (21,22,12), and blood which is free of microaggregates might be of valuable improvement over current practice.

1.3 General research strategy of this laboratory. In view of these considerations, we have developed a 2-pronged strategy in attempting to improve liquid preservation of erythrocytes. Our short-term aim is to help to provide the data required for early implementation of CPD-adenine solution so that patients may begin to share the benefits of the developments which have already been made. At the

same time, recognizing that better 2,3-DPG preservation is an important goal, we are pushing forward with the development of more advanced preservative systems which may combine the ability to maintain viability of red cells for 5 to 6 weeks, with improved 2,3-DPG levels.

In carrying out these investigations, increasing consideration has been given to the trend toward fractionation of blood into various formed elements and plasma components. Thus, we have paid considerable attention to the problem of packed erythrocyte storage, as contrasted with the storage of whole blood.

2. Progress to date and current clinical status of ongoing investigation

2.1 ^{51}Cr viability studies on human volunteers with blood stored in CPD-adenine.

2.11 Background

The use of CPD (citrate-phosphate-dextrose) as a standard medium for preservation of blood in vitro has been routine in conventional blood banking for several years. Even though the levels of 2,3-DPG in CPD stored blood are higher during the initial portion of the storage period than those stored in ACD, the levels of ATP are no better and perhaps lower than that in blood preserved with ACD. To prolong the life of red cells during storage, better maintenance of ATP is thought to be necessary. This has been possible by addition of adenine to give a final concentration of .25 mM and of more glucose to conventional

CPD (23,24). Since previous studies in this laboratory had confirmed the adequacy of this medium for a full 35 days of preservation with respect to ATP levels, it was necessary to proceed to the next phase of the study, i.e. the use of human volunteers to demonstrate that the 24 hour viability after reinfusion was also satisfactory.

2.12 Studies carried out

This laboratory served as a participant in the collection of viability studies in human volunteers with blood preserved in this new medium, CPD II, with a cooperative group under the IND filed with the FDA by Fenwal Laboratories. As the results in appendix I show, a total of 18 volunteers who met the usual AABB standards were studied (appendix Ia) in our laboratory. Of these, 8 were studied with packed cell units and the other 10 with whole blood units. All the units of whole blood stored in CPD II had 24 hour viability of at least 72% at the end of 35 days of storage. However, the preliminary studies with 5 packed cell units showed that 2 of these 5 packed cell units (#2 & 4 in table) did not have adequate glucose (and hence ATP) at the end of 35 days. Therefore, at the first meeting of the members of cooperative study group, it was mutually agreed to study the subsequent packed cell units at the end of 28 days. At the end of this period, all three remaining packed cell units showed better than 75% viability at 24 hours. These results have been sent to the central repository as required in the protocol.

Other phases of the study, i.e. results of massive transfusion of blood preserved with CPD II autologous transfusions and the yield of cryoprecipitates have not been included in the study at this institution because of the different nature of the patient population.

2.2 Studies with BAGPM

2.21 Background

BAGPM is a bicarbonate-containing preservative medium designed in this laboratory for the purpose of maintaining viability of packed red cells in a superior fashion. The system depends upon buffering of the stored cells with bicarbonate with loss of CO_2 from the system. This can be accomplished either by the use of a very permeable plastic film for the manufacture of bags, or an internal absorbing system. Our investigations suggest that calcium hydroxide, either in small sialastic bags ("baggies") or embedded in sialastic blocks ("blockies"), might be suitable internal CO_2 absorbing devices.

In earlier studies it was demonstrated that $Ca(OH)_2$ added to blood produced marked hemolysis. Hence, leakage of calcium hydroxide from the sialastic membrane "baggie" constituted a serious potential hazard. Therefore, the use of calcium hydroxide embedded sialastic blockies of 3 x 3 x 1 cm size were studied as replacements of the old baggies.

2.22 Studies with "baggies" and with "blockies" in which calcium hydroxide is embedded. Studies were carried

out with varying amounts of calcium hydroxide: baggies containing 6 gm, 3 gm and 1 gm calcium hydroxide were tested and it was found that the most satisfactory amount to provide adequate preservation of 2,3-DPG without compromising the levels of ATP, was in the 3-6 gm range (appendix IV). Blockies containing 6 g $Ca(OH)_2$ were placed into a standard blood bag containing ACD A. It was observed initially that calcium hydroxide shed from the raw surfaces when stored at room temperature in ACD and to some extent also in CPD. These difficulties were accentuated after autoclaving in ACD. They were overcome by providing a coating of liquid sialastic over the surface of the "blockie" and then enclosing it in another sialastic membrane. This prevented leaching of calcium hydroxide from the raw surfaces without affecting the CO_2 entrapment function of the blockie. After collection of blood and removal of plasma, BAGPM was added to the packed cells. As indicated by the accompanying data (appendix II, Fig. 1), a "blockie" containing 6 g $Ca(OH)_2$ proved to be a very satisfactory way to trap carbon dioxide generated from the bicarbonate buffer of the BAGPM medium. Using this system, pH levels were remarkably stable throughout the 42 day period of storage. At the end of storage, 2,3-DPG levels were 92% of the original levels, while approximately 62% of initial ATP levels were maintained. "Blockies" with 4 gm calcium hydroxide were also tested. However, it was noted that when only 4 gms of calcium hydroxide was employed, 2,3-DPG levels

were not maintained unless the bags were very frequently mixed. Hence, it appears that 6 gms of calcium hydroxide is the optimum amount. It is of interest to note that the blockies were also superior to the baggies in the maintenance of 2,3-DPG and ATP for the full 42 days of storage whether the units were mixed once a week or five times a week. The results of these studies are currently in press (appendix II).

2.23 Microaggregate formation and their importance

Microaggregate formation in BAGPM units with baggies or blockies had been previously shown to be markedly diminished (25). These data were further confirmed by the measurement of screen filtration pressure in all the units studied. Because of the marked diminution in the content of white cells, platelets, and fibrinogen, the interaction of these elements is probably much less in these units and therefore, the screen filtration pressure remains much lower (appendix III, Fig. 1) than in conventional blood units stored in ACD, CPD or CPD II-adenine. As the accompanying figure shows (appendix III, Fig. 2) whenever screen filtration pressure rose, it could be returned to normal by filtration through the usual blood filter utilized in the administration of blood to the patients. Therefore, it is anticipated that whatever microaggregates form, they would be readily cleared by the standard large-pore size blood filters conventionally used in the blood administration to humans.

2.24 Studies with thin-walled bags

The conventional PVC bags utilized in current blood banking are not adequately permeable to carbon dioxide to be lost from BAGPM at an adequate rate. Therefore, in addition to testing the internal carbon dioxide trap in the form of a blockie, we were interested in carrying out studies with a bag having five times or greater permeability for the carbon dioxide than the conventional PVC bag. With the collaboration of Jet Propulsion Laboratory in Pasadena, a bag with a wall thickness of 3¼ mil was designed and showed promising results in preliminary studies (Appendix V). Red cells maintained very satisfactory levels of ATP throughout 42 days of storage and lost 2,3-DPG only very slowly. The screen filtation pressure (SFP) remained low within 15 to 30 mm of mercury, almost the SFP of the fresh blood. However, similar promising results could not be obtained with the initial 2 bags made of slightly thicker thickness (6 mil) (Appendix VI) with respect to 2,3-DPG levels: Subsequent batches of 3 mil and 6 mil were equally ineffective (Appendix VII).

VIABILITY DATA ON CPD II

Whole Blood Units

	Hemoglobin (mg/dl)		Plasma Glucose (mg/dl)		K+ (Meq/L)		Na+ (Meq/L)		(μMo
No.	Day 0	Day 35	Day 0	Day 35	Day 0	Day 35	Day 0	Day 35	Day 0
1	6	63	470	230	4	27	170	155	3.69
2	10	38	476	274	4	29	170	154	4.03
3	9	54	475	256	5	32	170	152	4.35
4	6	40	474	238	4	22	165	156	3.24
5	8	13	465	265	4	26	166	157	6.08
6	7	34	504	288	4	36	169	155	3.94
7	12	74	494	241	4	29	171	156	4.04
8	12	53	522	281	4	26	170	156	4.81
9	16	34	506	250	4	27	171	159	4.65
10	106	17	450	266	6	17	165	161	4.58

Packed Cell Units

1	1	873	483	0	3	93	169	102	4.35
2	0	1102	515	4	4	86	174	111	4.18
3	4	378	518	20	4	75	165	116	4.80
4	2	224	514	0	4	93	170	104	4.90
5	0	569	493	46	4	73	171	120	3.83

No.	Day 0	Day 28	Day 0	Day 28	Day 0	Day 28	Day 0	Day 28	Day
6	0	106	455	104	4	81	168	130	4.48
7	3	81	446	66	4	59	168	145	4.70
8	0	137	447	2	3	98	169	130	4.90

*At the end of 35 days of storage, the units with negligible amount of gluco Hb ATP had either marginal (#1) or in 24 hou viability of red blood cells.

VIABILITY DATA ON CPD II BLOOD

Appendix I

Whole Blood Units

K+ (Meq/L)		Na+ (Meq/L)		ATP (µMoles/g Hb)		Erythrocytes 2,3-DPG (µMoles/g Hb)		24 Hou Viabilit
)	Day 35	Day 0	Day 35	Day 0	Day 35	Day 0	Day 35	Day 35
	27	170	155	3.69	1.99	14.44	1.57	84.81
	29	170	154	4.03	2.71	13.68	1.14	77.63
	32	170	152	4.35	2.48	12.61	1.24	72.08
	22	165	156	3.24	2.99	18.47	1.09	89.35
	36	166	157	6.08	3.27	14.15	0	73.76
	36	169	155	3.94	2.80	12.33	0.10	85.71
	39	171	156	4.04	2.15	12.51	0.27	74.65
	26	170	156	4.81	2.41	13.30	0.11	83.27
	27	171	159	4.65	3.27	12.99	0.11	82.63
	17	165	161	4.58	2.80	22.01	0	89.67

Packed Cell Units

93	169	102	4.35	1.28	14.05	0.06	72.50*
86	174	111	4.18	1.66	14.29	0	54.50*
75	165	116	4.80	2.39	14.29	0.12	77.97
93	170	104	4.90	1.71	11.80	0	53.28*
73	171	120	3.83	2.31	18.72	0.21	75.67
0 Day 28	Day 0	Day 28	Day 0	Day 28	Day 0	Day 28	Day 28
.81	158	130	4.48	2.27	16.20	1.69	83.58
59	168	145	4.70	4.29	12.72	0.49	91.54
98	169	130	4.90	3.20	15.24	0.50	82.67

the end of 35 days of storage, these three packed cell
its with negligible amount of glucose & <2.00 µMoles/g
ATP had either marginal (#1) or inadequate (#2 & 4)
hou viability of red blood cells.

Appendix Ia

Clinical Protocol

Modified Anticoagulant Citrate Phosphate Dextrose Solution with Adenine (CPD-II)

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CLINICAL PROTOCOL

Modified Anticoagulant Citrate Phosphate Dextrose Solution with Adenine (CPD II)

INTRODUCTION

General

In view of the recent emphasis on conservation of blood as a national resource, initiation of clinical trials of a blood anticoagulant-preservative permitting liquid storage beyond 21 days appears appropriate at this time. Extension of red cell shelf-life will decrease wastage, most significantly in less populous areas of the United States, and during an international conflict or emergency. It is probable that future research will render obsolete any formula selected now; however, current knowledge of red cell preservation is sufficiently advanced to extend safely to 35 days the red cell storage limitation imposed for over 30 years. This knowledge has been reviewed recently (1,2,3,4), and from these summaries certain concepts emerge which are relevant to the design of this protocol.

Applicability of ACD Data

Information exists concerning the safety of ACD-A supplemented with adenine. Since CPD is only a slight modification of ACD-A, this data base is applicable as background for suitability of the proposed anticoagulant.

Safety of Adenine

Adenine as an additive for human use is safe, particularly at the concentration proposed. Not only have the Swedish and German clinical experiences with double the proposed concentration been apparently uneventful, but recent human studies in the United States found no compromise of renal function at acute doses of 10 mg per kilogram (5). Despite the safety of adenine suggested by European use, its documentation is limited. Therefore, documentation of safety is an integral part of this protocol. It is particularly important in patients subjected to massive acute transfusions, in those with intensive chronic transfusion requirements, and in patients with pre-existing renal disease. More complete safety documentation is presented in Appendix B of this protocol.

Therapeutic Equivalency

Available data suggest that hemoglobin oxygen affinity would be at least no greater at the end of 35 days of red cell storage in the proposed anticoagulant than at the end of 21-day storage in CPD or ACD-A. Although it is suspected that red cells with lower oxygen affinity may be desirable in selected clinical settings, no proven

stable additive which would reduce oxygen affinity during storage is presently available. Thus, for purposes of therapeutic equivalency, survival of stored red cells 24 hours after injection remains the most appropriate in vivo measurement.

Components

Negligible data have been published regarding the effects of either adenine or increased glucose concentration on yields or storage characteristics of components commonly made and dispensed by blood banks throughout the country. Yields should be evaluated in platelet preparations and Cryoprecipitated Antihemophilic Factor.

Multiple Institution Study

It is clear that numerous investigators have competence to perform clinical studies of improved liquid red cell storage. In order to enlist their expertise and obtain data on a diverse patient base, multiple institutions will participate in clinical trials. This will also expedite data acquisition. Each participating investigator and the parent institution must agree to follow the restrictions, methods and reporting techniques specified in this protocol. At the same time, certain aspects of the protocol are broadly drawn to permit investigators to comply with individual institutional constraints on human use. Certain investigators may choose to concomitantly obtain data not required by this protocol. This is specifically permitted, provided the requirements of the protocol are met. Those protocol provisions which follow are intended to serve as a master under which each investigator would develop more detailed study plans. Only those data have been sought which the authors regard as critical to document the safety and equivalency of the proposed anticoagulant. Further, although as stated, we consider historical controls of ACD and CPD adequate, parallel controls may be obtained by particular investigators for a particular aspect of their studies.

GENERAL OPERATIONAL REQUIREMENTS

Task Force Participation

In order to insure organized, systematic data acquisition and analysis, a task force is proposed. The coordinators will be the Blood Research Division, Letterman Army Institute of Research and Fenwal Laboratories, Division of Travenol Laboratories, Inc. Other qualified investigators will be added to fulfill the data requirements. Each participant must agree in writing to conform to the provisions of this protocol. The suppliers of the containers for blood collection shall have the right to refuse to supply any participant who does not demonstrate satisfactory compliance.

Record of Task Force Participants

The Blood Research Division, Letterman Army Institute of Research, will maintain a registry of task force participants and the phase(s) in which they are active. The Bureau of Biologics and all participants will be supplied with a list of fellow workers, which will be updated upon each entry or withdrawal of participants.

Periodic Meetings

Meetings of task force participants will be held periodically, but no less than annually from the date of commencement of the studies, when it is mutually agreed that such a meeting will further the orderly progression of the studies.

Data Repositories

In order to permit independent analysis of data, two repository agents will be established for those studies utilizing containers manufactured by Fenwal Laboratories: the Blood Research Division in conjunction with the Department of Informational Sciences, Letterman Army Institute of Research, and Fenwal Laboratories. All requisite records, forms, and clinical reports will be prepared in triplicate, one of which may be by electronic reproduction, and one each of which will be forwarded to each of the above data repositories. Progress reports of the task force efforts will be prepared at appropriate intervals jointly by the data repository agents. A copy will be supplied each participant, and the Bureau of Biologics (BoB) for their information and review.

Adverse Reaction Notification

If an unexpected severe adverse reaction is encountered by any investigator, a telephonic report as to the nature and severity of the adverse reaction will be filed with both repository agents. Notification to the Bureau of Biologics will be provided by the manufacturer as required by the regulations. Determination of what constitutes a severe adverse reaction will be the responsibility of those members of the task force with direct medical responsibility for the infusion resulting in the reaction.

Proposed Anticoagulant-Preservative Formula

The generic name of the proposed anticoagulant is "Modified Anticoagulant Citrate-Phosphate-Dextrose Solution with Adenine." The trivial name will be "CPD-II," and will have the following formula:

Each 63 ml of anticoagulant contains 206 mg Citric Acid (hydrous) U.S.P., 1.66 g Sodium Citrate (hydrous) U.S.P.,

140 mg Sodium Biphosphate, N.F., 2.01 g Dextrose (hydrous) U.S.P., and 17.3 mg Adenine. 63 ml Modified Anticoagulant CPD Solution with Adenine is for collection of 450 ml whole blood.

At 22°C, the pH of the anticoagulant solution is approximately 5.61. The final concentration of adenine in 450 ml of whole blood in the preservation is 0.25 mM, and the final concentration of dextrose is approximately 350-400 mg per dl, depending upon the donor plasma concentration at the time of phlebotomy.

Blood Containers

All blood and blood components for all phases will be drawn into, prepared in, and stored in polyvinyl chloride plastic bags (PL 146). In those studies requiring controls of conventionally anticoagulated blood, Anticoagulant Citrate Phosphate Dextrose Solution (CPD) in currently approved blood collection systems will be used. All containers with anticoagulant, integral tubing, and satellite bags will be sterile and pyrogen-free.

Donors

All homologous donors must meet published standards of the BoB, the American Red Cross, or the American Association of Blood Banks. All normal persons volunteering to receive autologous blood infusions after storage will meet those standards which are for the protection of the donor. Normal volunteers will only be accepted for study after a physical examination by, and written approval from a licensed physician. All patients from whom blood is drawn as part of an autologous predeposit program must meet the standards for such donors published by the American Association of Blood Banks.

Human Use

During any and all aspects of clinical trials, the provisions of AR 70-25, Use of Volunteers as Subjects of Research, and Protection of Human Subjects, Federal Register, Volume 39, No. 105, May 30, 1974, will apply and be adhered to strictly by investigators. No persons will be transfused with any blood component under this protocol unless informed consent witnessed by other than the counseling physician has been obtained prior to infusion. If the patient is incompetent or unable to consent, informed consent must be obtained from a parent or guardian. A copy of all executed informed consent forms will be retained by the investigator under whose supervision the infusion was conducted.

Components

All components will be defined as in the Standards of the American Association of Blood Banks or BoB.

Storage

Except for duration, storage of whole blood and red blood cells will be in accordance with the Standards of the American Association of Blood Banks or BoB.

Labels

All requirements for labeling of blood defined in the Standards of the American Association of Blood Banks and BoB will be followed.

Preinfusion Tests

Preinfusion compatibility testing of homologous blood will be in accordance with Standards of the American Association of Blood Banks, by methods shown to be at least as good as those defined in the "Technical Methods and Procedures" of the American Association of Blood Banks, 7th Edition, 1974.

CLINICAL STUDY GROUPS

General

Since diverse studies will be required to document adenine safety, equivalency of red cells undergoing extended storage, and component recovery and yield, data acquisition will be facilitated by assigning subjects to clearly defined phases. Some investigators may not wish to participate in all phases. Except for Phase IA, numbering of phases does not imply that they will be conducted sequentially, as studies from Phases IB, II, III and IV may be conducted concurrently by a particular investigator. Phase IA will be completed, and the data analyzed to assure acceptable survival (as defined in Appendix C), prior to commencement of any *in vivo* studies in other phases.

I. Phase I (Autologous Studies)

Autologous transfusions will be studied in Phase I.

A. Study Design - Phase IA.

Phase I, Group A (Autologous Red Blood Cell Survival) will be composed of normal volunteers. The primary objective of this study group will be to document

equivalency of postinfusion survival of red cells prepared from blood drawn in CPD-II. Documentation of adenine safety is not a primary objective since the volumes reinfused will be insufficient to obtain meaningful toxicity data, and plasma will have been largely removed by washing.

1. General

To establish red cell survival characteristics of CPD-II, no less than three, nor more than five, laboratories will participate. Each laboratory will complete autologous survival studies, and in vitro chemical analyses, on blood from at least fifteen (15) volunteers, of whom at least five (5) shall be non gravid female. Computation of these minimal study numbers are detailed in Appendix C. Assay techniques will be at least as good as those outlined in Appendix D.

2. In Vitro Tests of Normal Volunteers

In addition to tests required to determine whether a candidate meets the donor Standards, the following tests will be performed prior to phlebotomy to obtain the study unit:

The day before

a. hematocrit

b. hemoglobin

c. red cell count

d. red cell indicies (MCV, MCHC, MCH)

e. reticulocyte count

f. examination of peripheral smear

g. hemoglobin electrophoresis

h. pregnancy test in all females

Examine i. G-6-PD (Glucose-6-Phosphate Dehydrogenase)

Significant abnormality of any of the above studies or a positive pregnancy test in the case of females, shall exclude a donor as a normal volunteer for red cell survival studies.

3. Samples

In order to minimize risks of contamination, whole blood will be drawn into triple integral bag systems. Within one hour of phlebotomy, at least five units will be packed to an hematocrit of 75% ± 5% by each participant laboratory. By at least three investigators, at least three units of either packed red cells or whole blood will be subjected to transportation trauma on the 28th day of storage by shipment to a second investigator in Phase IA, who will reship to the city of origin for preinfusion analysis and survival studies.

4. In Vitro Analysis of Stored Red Blood Cells and Whole Blood

On day zero and on day 35, the following values will be determined on each stored unit:

a. Supernatant plasma:

- hemoglobin — benzidine method
- glucose — hexokinase method
- potassium, sodium — flame photometer
- pH and temperature of determination —

b. Red cells:

- hematocrit
- 2,3-DPG
- ATP

In addition, an aliquot obtained subsequent to the 32nd day will be cultured for both anaerobic and aerobic bacteria at 4° and 37°C to determine sterility. Sterility will be documented prior to reinfusion.

5. Survival Studies

On the 35th day of storage, an aliquot of red cells will be labeled with 51-Cr and infused into the volunteer donor. Appropriate venous samples will be obtained postinfusion to determine the red cell survival at 24 hours after infusion.

6. Data Reporting

Information gathered in Phase I, Group A will be recorded on CPD-II Form A (Appendix A). In addition to the percent survival at 24 hours, a graphic display of all points used to calculate survival will be submitted to the repository agents. Units subjected to trauma of transportation will be identified as such in the "comments" section of the form. Copies will be forwarded to each repository agent within fifteen (15) days of completion of the survival studies.

B. Study Design - Phase IB

Phase I, Group B (Autologous Transfusion) will be composed of patients scheduled to undergo elective surgery who have predeposited autologous units of whole blood and/or components anticoagulated with CPD-II for reinfusion at surgery or in the postoperative period. The primary objective is to document clinic safety and equivalency in patients receiving blood and components stored for graded intervals. Because of blood volume instability in this group, survival studies are inappropriate, and normal volunteers in Phase IA must be utilized to reliably determine red cell survivals.

1. General

To establish clinical response to, and possible adverse effects of autologous blood stored in CPD-II, studies from no less than twenty (20) patients will be completed by each Phase I, Group B participant. Although ideally only autologous red cells and components would be transfused, this may not be possible in all patients. Preferably, all homologous red cells and components would be prepared and stored in CPD-II; however, this also may not be practical or possible. Should patients in Phase I, Group B receive homologous red cells or components, data will be gathered on each unit without regard to

anticoagulant as though the unit had been drawn and stored in CPD-II. The data repository agents will mutually determine whether the number of homologous units given will require reclassification of a particular patient into Phase II or III.

2. Infusion Products

Decisions regarding number of units, timing of phlebotomies, and whether to store as packed cells or whole blood will be the sole responsibility of the participant and cooperating clinicians.

3. Patient Summary (CPD-II Form I)

Relevant data summarizing the experience of each patient for the period included in the study shall be recorded on CPD-II Form I (Appendix A), which is self-explanatory as to the information sought. Only one summary is required for each discrete study period.

4. Unit Infusion Data (CPD-II Form II)

Relevant data regarding each unit shall be recorded on CPD-II Form II (Appendix A), which is self-explanatory regarding the information sought. A separate page of this form must be completed for each donor unit or component pool transfused, without regard as to whether such was prepared and stored in CPD-II.

5. Clinical and Laboratory Data (CPD-II Form III)

Relevant clinical and laboratory data shall be recorded on CPD-II Form III (Appendix A). The information required is indicated by the bold delineated blocks, labeled "PRE," "POST" and "DCHG." The "PRE" data should be that obtained most recently prior to the infusion, the timing of which will vary among patients. The "POST" data will be obtained between 24 and 48 hours postinfusion. The "DCHG" data will be that obtained from inpatients most nearly corresponding with their discharge providing 48 hours has elapsed since the "POST" data was obtained. The unaccented intervening blocks may be filled in with other appropriately timed tests, but are optional. If an autopsy is performed, the cause

of death and the results of histologic examination of the kidneys by conventional and cross polarized light microscopy shall be included under "comments."

6. Autologous Unit Verification

All containers for blood or components intended for later autologous reinfusion shall bear a special label as defined in the standards for autologous transfusion of the American Association of Blood Banks. Prior to reinfusion, the donor-recipient, if able, shall verify in writing that he (she) has identified his (her) signature on the special label. Such verification, or a certification by a physician of the person's inability, shall be part of the permanent records of the investigator responsible for the infusion.

7. Data Reporting Period

Data on each recipient shall be forwarded to both data repository agents within thirty (30) days following the final infusion reported.

II. Phase II (Conventional Transfusion Requirements)

All candidates in this phase will be patients, and it is anticipated this will comprise the largest grouping. In this phase, medical and surgical patients, both adult and pediatric, will be infused with homologous whole blood and components in response to requirements for 5 or less donor units, or proportionately less in the case of pediatric patients. The primary objective of the phase is to document clinical safety and equivalency of CPD-II. No patients will be excluded due to underlying disease. No efforts will be made to give an individual patient products of uniform storage interval; variable storage will frequently be represented in each candidate.

Study Design, Phase II

A. General

In order to establish clinical response to, and possible adverse effects of, homologous blood stored in CPD-II, diverse patient types will be studied in Phase II. The number of patients to be entered into this phase is open-ended, with final requirements to be determined by continuing data analysis of patients

studied. All decisions regarding storage duration, acceptance or rejection of a particular unit for infusion, the number of units, the type of units, and the rate of infusion shall be at the sole discretion of the investigator and his cooperating physicians, and will be guided by the dictates of good medical practice, and the constraints of human use by the participating institution. It is probable that many patients will receive blood products which have been anticoagulated with other than CPD-II. Data must be gathered on all infusions as though all had been anticoagulated in CPD-II.

B. Patient Summary (CPD-II Form I)

Relevant data summarizing the experience of each patient for the period included in the study shall be recorded on CPD-II Form I (Appendix A) which is self-explanatory as to the information sought. Only one summary is required for each discrete study period.

C. Unit Infusion Data (CPD-II Form II)

Relevant data regarding each unit infused shall be recorded on CPD-II Form II (Appendix A), which is self-explanatory as to the information sought. A separate page of this form must be completed for each donor unit or discrete component pool transfused without regard to anticoagulant.

D. Clinical and Laboratory Data (CPD-II Form III)

Relevant clinical and laboratory data shall be recorded on CPD-II Form III (Appendix A). The information required is indicated by the bold delineated blocks, labeled "PRE," "POST" and "DCHG." The "PRE" data should be that obtained most recently prior to the infusion, the timing of which will vary among patients. The "POST" data will be obtained between 24 and 48 hours postinfusion. The "DCHG" data will be that obtained from inpatients most nearly corresponding with their discharge providing 48 hours has elapsed since the "POST" data was obtained. The unaccented intervening blocks may be filled in with other appropriately timed tests, but are optional. If an autopsy is performed, the cause of death and the results of histologic examination of the kidneys by conventional and crossed polarized light microscopy shall be included under "comments."

E. Data Reporting Period

Data on each recipient shall be forwarded to both data repository agents within thirty (30) days following the final infusion reported.

III. Phase III (Massive Transfusion Requirements)

All candidates in this phase will be patients, and all will be transfused with homologous blood and components in response to requirements for greater than 5 donor units. The primary objective of this phase is to document clinical safety of blood and components at extreme transfusion requirements.

A. Phase III, Group A

Phase III, Group A will be patients transfused due to massive acute requirements. Examples would be severe trauma, acute bleeding due to a coagulopathy, or exchange transfusion. Large numbers of patients may be difficult to accumulate due to the problems associated with obtaining informed consent.

B. Phase III, Group B

Phase III, Group B will be patients with large chronic requirements. Examples would be chronic uncompensated anemia, thrombocytopenia, and lifelong bleeding disorder prophylaxis. It is anticipated that principally components and little whole blood will be given to these patients. The objective is to document safety of repeated small doses of adenine.

C. Study Design - Phase III

1. General

In order to establish clinical response to, and possible adverse effects of, homologous blood stored in CPD-II, diverse patient types will be studied in Phase III. The number of patients to be entered into this phase is open-ended, with final requirements to be determined by continuing data analysis of patients studied. All decisions regarding storage duration, acceptance or rejection of a particular unit for infusion, the number of units, the type of units, and the rate of infusion shall be at the sole discretion of the investigator and his cooperating physicians, and

will be guided by the dictates of good medical practice and the constraints of human use by the participating institution. It is probable that many patients will receive blood products which have been anticoagulated with other than CPD-II. Data must be gathered on all infusions as though all had been anticoagulated in CPD-II.

2. Patient Summary (CPD-II Form I)

Relevant data summarizing the experience of each patient for the period included in the study shall be recorded on CPD-II Form I (Appendix A), which is self-explanatory as to the information sought. Only one summary is required for each discrete study period.

3. Unit Infusion Data (CPD-II Form II)

Relevant data regarding each unit infused shall be recorded on CPD-II Form II (Appendix A), which is self-explanatory as to the information sought. A separate page of this form must be completed for each donor unit or discrete component pool transfused without regard to the anticoagulant.

4. Clinical and Laboratory Data (CPD-II Form III)

Relevant clinical and laboratory data shall be recorded on CPD-II Form III (Appendix A). The information required is indicated by the bold delineated blocks, labeled "PRE," "POST" and "DCHG." The "PRE" data should be that obtained most recently prior to the infusion, the timing of which will vary among patients. The "POST" data will be obtained between 24 and 48 hours postinfusion. The "DCHG" data will be that obtained from inpatients most nearly corresponding with their discharge providing 48 hours has elapsed since the "POST" data was obtained. The unaccented intervening blocks may be filled in with other appropriately timed tests, but are optional. If an autopsy is performed, the cause of death and the results of histologic examination of the kidneys by conventional and crossed polarized light microscopy shall be included under "comments."

5. Data Reporting Period

Data on each recipient shall be forwarded to both repository agents periodically, but no less frequently than every six months.

IV. Phase IV (Components)

Although not strictly a clinical study, information regarding yields and storage characteristics of platelets, and yields of Cryoprecipitated Antihemophilic Factor may be gathered in conjunction with each of the above phases. Removal of platelets and/or cryoprecipitate from units primarily intended to meet red cell requirements is widely practiced by American blood banks. To manipulate similarly units stored and infused in other phases of this study is appropriate, to as closely as possible duplicate actual current practices. Thus, Phase IV studies will be conducted concomitantly on units prepared and stored for other phases of this protocol.

Study Design - Phase IV

A. General

To establish yields and storage behavior of platelets, and yields of Cryoprecipitated Antihemophilic Factor, no less than twelve (12) donor units of platelet concentrate and/or cryoprecipitate will be evaluated by each participant in Phase IV. Study methods shall be at least as good as those outlined in Appendix D.

B. Unit Component Data (CPD-II Form B)

Information gathered under Phase IV shall be entered on CPD-II Form B (Appendix A), which is self-explanatory as to the data required. Portions of the form which do not apply to the component studied should be left blank. A separate sheet must be used for <u>each</u> donation.

C. Data Reporting

Accumulated component data will be forwarded to both data repository agents at appropriate intervals but no less frequently than every ninety (90) days.

GENERAL REFERENCES

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3. Minutes of Fenwal Blood Preservation Meeting. Travenol Laboratories, Morton Grove, Illinois, January 14, 1974.

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5. Roth, G.J., Moore, G.L., and Poskitt, T.R. Renal effects of intravenous adenine in man. Clin. Res. 22:322A, 1974.

APPENDIX A

Forms

The following five forms are used for data reporting.

CPD-II FORM A
RED CELL SURVIVAL DATA
USE ONE SHEET FOR EACH STUDY

CODE ☐☐☐☐

VOLUNTEER IDENTIFICATION:

NAME _____ SSN ☐☐☐ ☐☐ ☐☐☐☐

RACE: ☐ CAUCASIAN ☐ BLACK ☐ OTHER _____

AGE: ☐☐ YEARS SEX: ☐ MALE ☐ FEMALE WEIGHT: ☐☐☐ LBS

PREPHLEBOTOMY DONOR DATA:

HEMATOCRIT: ☐☐.☐ % HEMOGLOBIN: ☐☐.☐ g/dl MCV: ☐☐☐ μ^3

RED CELL COUNT: ☐.☐☐ x $10^6/\mu l$ RETICULOCYTES: ☐☐.☐ % MCH ☐☐ µµg

G-6-PD:SCREEN: ☐ NEGATIVE ☐ POSITIVE MCHC: ☐☐ %

PERIPHERAL MORPHOLOGY: _____

UNIT DATA: BLOOD BAG CODE ☐☐☐☐☐☐☐ LOT NUMBER ☐☐☐☐☐☐☐☐☐

SUPERNATANT PLASMA:	DAY 0	DAY 35
HEMOGLOBIN	☐☐.☐ mg/dl	☐☐.☐ mg/dl
GLUCOSE	☐☐☐.☐ mg/dl	☐☐☐.☐ mg/dl
POTASSIUM	☐☐.☐ mEq/l	☐☐.☐ mEq/l
SODIUM	☐☐☐ mEq/l	☐☐☐ mEq/l
pH (AT ☐☐ °C)	☐.☐☐	☐.☐☐
RED CELLS:		
HEMATOCRIT	☐☐.☐ %	☐☐.☐ %
2,3-DPG	☐☐.☐ µM/g Hb	☐☐.☐ µM/g Hb
ATP	☐☐.☐ M/g Hb	☐☐.☐ M/g Hb

STERILITY CONFIRMATION
NEGATIVE

AEROBIC 4°C ☐

ANAEROBIC 4°C ☐

AEROBIC 37°C ☐

ANAEROBIC 37°C ☐

CLINICAL INFUSION RESPONSE:	PREINFUSION	1-4 HOUR	24 HOUR
PULSE PER MIN	☐☐☐	☐☐☐	☐☐☐
TEMPERATURE (C)	☐☐☐.☐	☐☐☐.☐	☐☐☐.☐
BLOOD PRESSURE (mm Hg)	☐☐☐/☐☐☐	☐☐☐/☐☐☐	☐☐☐/☐☐☐
URINE Hb	☐ NEG ☐ POS	☐ NEG ☐ POS	☐ NEG ☐ POS

24-HOUR RED CELL SURVIVAL DATA:

COMMENTS: _____

INSTITUTION ☐☐ INVESTIGATOR'S SIGNATURE ☐☐

DATE ☐☐ ☐☐ ☐☐ ☐☐

CODE ☐☐☐☐

CPD-11 FORM B

UNIT COMPONENT DATA

USE SEPARATE SHEET FOR EACH UNIT

DONOR INFORMATION:

NAME _____ SSN ☐☐☐☐☐☐☐☐☐

RACE: ☐ CAUCASIAN ☐ BLACK ☐ OTHER _____

AGE: ☐☐ YEARS SEX: ☐ MALE ☐ FEMALE WEIGHT: ☐☐☐ LBS

HEMATOCRIT: ☐☐.☐ % HEMOGLOBIN: ☐☐.☐ g/dl RETICULOCYTES: ☐☐.☐ %

PLATELET COUNT: ☐☐☐ x 10^5 FACTOR VIII: ☐☐☐ Iu/ml FIBRINOGEN ☐☐☐ mg/dl

COLLECTION DURATION ☐☐ MINUTES ☐ VACUUM ☐ GRAVITY

PLATELET RECOVERY:

BAG WT: ☐☐.☐ gms - TARE ☐☐.☐ gm = VOLUME ☐☐.☐ ml x 10^3

x UNIT PLATELET CT. ☐.☐☐ x $10^6/\mu l$ = ☐.☐☐ x 10^{10} PLATELETS PER UNIT

PLATELET STORAGE: ☐☐ °C TEMPERATURE AGITATION ☐ YES ☐ NO MOVEMENTS/MIN ☐☐

	0	24	48	72
COUNT (x $10^6/\mu l$)	☐.☐☐	☐.☐☐	☐.☐☐	☐.☐☐
pH AT ☐☐ °C	☐.☐☐	☐.☐☐	☐.☐☐	☐.☐☐

CRYOPRECIPITATE:

FACTOR VIII: ASSAY METHOD _____

BAG WT. ☐☐.☐ g - TARE ☐☐.☐ g = VOLUME ☐☐.☐ ML

x FACTOR VIII ☐☐.☐ Iμ/ML = YIELD ☐☐☐ Iμ/DONOR UNIT

☐☐☐ %

FIBRINOGEN: ASSAY METHOD _____

FIBRINOGEN ☐☐☐ mg/dl x VOLUME $\frac{☐☐.☐}{100}$ = YIELD ☐☐☐ MG/DONOR UNIT

☐☐☐ %

COMMENTS: _____

INSTITUTION ☐☐ INVESTIGATOR'S SIGNATURE ☐☐

CPD-II FORM 1
PATIENT SUMMARY
USE ONE FORM FOR EACH PATIENT

☐☐☐☐ CODE

PATIENT IDENTIFICATION:

NAME _____ HOSP NO. ☐☐☐☐☐☐☐

RACE: ☐ CAUCASIAN ☐ BLACK ☐ OTHER _____

AGE: ☐☐ CALENDER UNITS ☐ YEARS ☐ MONTHS ☐ DAYS

WEIGHT: ☐☐☐ POUNDS ☐ MALE ☐ FEMALE

ENTERED STUDY ☐☐☐☐☐☐ (M M D D Y Y) TERMINATED STUDY ☐☐☐☐☐☐ (M M D D Y Y)

CLINICAL DATA:

REASONS FOR TRANSFUSION ☐ MEDICAL ☐ SURGICAL

DIAGNOSIS _____

IF SURGICAL, OPERATIVE PROCEDURES — MONTH DAY YEAR

1. _____ ☐☐ ☐☐ ☐☐
2. _____ ☐☐ ☐☐ ☐☐
3. _____ ☐☐ ☐☐ ☐☐

PRETRANSFUSION PROGNOSIS: ☐ GOOD ☐ POOR (REASON) _____

FOLLOW-UP: ☐ IMPROVED ☐ NOT IMPROVED ☐ DEATH

IF DEATH, WAS AN AUTOPSY PERFORMED? ☐ YES ☐ NO

TRANSFUSION SUMMARY:

TOTAL INFUSIONS (SPECIFY NUMBER OF DONOR UNITS OF APPROPRIATE PRODUCTS)

☐ WHOLE BLOOD ☐ WASHED CELLS ☐ CRYO-POOR FFP

☐ PACKED CELLS ☐ CRYOPRECIPITATE ☐ PLATELET CONCENTRATES

☐ FROZEN CELLS ☐ FRESH FROZEN PLASMA (FFP) ☐ OTHER _____

SUMMARY COMMENTS ON RESULTS OF ALL INFUSIONS, WITH CAUSE OF DEATH IF AUTOPSY PERFORMED: _____

INSTITUTION ☐☐ SIGNATURE ☐☐

DATE: ☐☐ ☐☐ ☐☐

CODE ☐☐☐☐

CPD-II FORM II

UNIT INFUSION DATA

USE SEPARATE SHEET FOR EACH UNIT

PATIENT NAME _____ HOSP NO. ☐☐☐☐☐☐☐☐

UNIT DATA:

DONOR/UNIT NO. ☐☐☐☐☐ BAG LOT NO. ☐☐☐☐☐☐☐☐☐☐☐☐

☐ AUTOLOGOUS ☐ HOMOLOGOUS

ANTICOAGULANT: ☐ CPD-II ☐ CPD ☐ OTHER _____

PRODUCT: ☐ WHOLE BLOOD ☐ WASHED CELLS ☐ FRESH FROZEN PLASMA (FFP)

☐ PACKED CELLS ☐ PLATELET CONC. ☐ CRYO-POOR FFP

☐ FROZEN CELLS ☐ CRYOPRECIPITATE ☐ OTHER _____

EXPIRATION DATE: ☐☐☐☐☐☐
M M D D Y Y

INFUSION RECORD:

THIS INFUSION WAS ☐☐ IN A SERIES OF ☐☐ OF TRANSFUSIONS

ML INFUSED ☐☐☐ DONOR UNITS INFUSED (POOLED COMPONENTS) ☐☐

DATE GIVEN ☐☐☐☐☐☐ TIME INFUSION STARTED ☐☐☐☐ HOURS
M M D D Y Y

DURATION OF INFUSION: ☐ <1 HOUR ☐ 1-3 HOURS ☐ >3 HOURS

INFUSION RESULTS:

Was there a significant change in pulse, respiration, temperature, or any other evidence of an acute adverse reaction associated with the infusion:

☐ NO ☐ YES

If yes, describe the nature of the adverse reaction, and enter the appropriate data on CPD-II Form III.

_____

_____

_____

_____

_____

_____

_____ _____

INSTITUTION ☐☐ SIGNATURE ☐☐

NAME ______________________ HOSP NO. [ ]

	TIME / TEMP	TIME / PULSE	TIME / BP	TIME / HBG/HCT	TIME / BUN	TIME / CREAT
y			/			
y y			/			
y y			/			
y y			/			
y y			/			
y y			/			
y y			/			
y y			/			

OPTIONAL ADDITIONAL STUDIES

TIME	TEST	RESULT	NORMAL/UNITS

INSTITUTION [ ][ ] ______________________ SIGNATURE [ ][ ]

APPENDIX B
Toxicity Summary - Adenine

The principal adverse effect in animals and man resulting from excessive adenine administration is nephrotoxicity (1,2,3,4). Nephrotoxicity is thought to be related to precipitation of the poorly soluble metabilite of adenine, 2,8 dihydroxyadenine (DOA), in the urinary tract (1). This metabolite is produced by the action of hepatic xanthine oxidase on adenine (4).

Animal Studies

Table I summarizes most published adenine toxicity studies in animals (1,4,5) as well as the extensive unpublished investigations of intravenously administered adenine by Garvin et al (2). Although the LD_{50} for rats and mice for intraperitoneally administered adenine is of the order of 200-300 mg/kg (1), the LD_{50} for intravenous adenine has not been determined because of solubility limitations.

Acute, intravenous administration of adenine, 50 mg/kg, causes neither renal dysfunction nor morphologic lesions in normal and dehydrated rats and mongrel dogs, although in one study a dose of 50 mg/kg administered as a single dose to rats did produce transient elevations of serum urea nitrogen and renal crystal formation. A dose of 25 mg/kg is well tolerated in unilaterally nephrectomized rats and normal Dalmatian dogs (2). Mild (<40 mg%) transient (24 hours) blood urea nitrogen elevation is the only abnormality noted at doses between 50 and 100 mg/kg in rats (2). Baboons given 25 mg/kg IV during hemorrhagic shock showed no differences in renal function upon recovery from shock when compared to non-adenine treated animals (6).

Subacute intravenous administration of adenine at 50 mg/kg/day for 14 days is well tolerated by normal rats, while 10 mg/kg/day for 15 days is well tolerated in Dalmatian dogs (8) as well as in Beagle dogs given 10 mg/kg/5-6X/week intravenously for three weeks (5). Monkeys (Macaca irus) showed no evidence of toxicity given adenine 10 mg/kg/day, IV, for five days (7).

No teratologic sequelae were found in pregnant Holtzman rats and New Zealand rabbits administered intravenous adenine, 50 mg/kg, during the 6-15th day of gestation (2).

Human Studies

Kinetic studies in man have shown that intravenous adenine disappears rapidly from the circulation with an elimination half-life of 15-25 minutes (9,10). DOA binds to plasma proteins thereby inhibiting precipitation (19). During the first six hours post infusion, approximately 15% of the injected dose appears unchanged in the urine, whereas 4% is excreted as DOA and 3% as 8-

hydroxyadenine (9,10). While much of the DOA recovered in the urine following a 10 mg/kg IV injection appears in crystalline form, the mere presence of DOA crystals at this adenine dosage has not been shown to be associated with renal toxicity (9).

Brief summaries of six human studies in which adenine was administered orally or intravenously appear in Table II (3,9-13). In addition, there is evidence that tens of thousands of Swedish and German patients have received adenine preserved blood (at twice the adenine concentration proposed in this IND) without report of serious toxicity (11,13-15).

Only one case of proven adenine toxicity has been reported (3). In this patient, approximately 71.4 mg/kg* per day for five days was administered orally in a misguided attempt to treat pernicious anemia. The patient developed severe uremia but recovered almost completely (residual blood urea nitrogen of 38 mg% was the only abnormality) (3).

Numerous studies attest to the safety of acute intravenous administration of adenine to humans in doses up to 15 mg/kg (equivalent to 60 units of CPD-II) (8,9,11-17). No immediate or long term (five year follow-up) toxicity has been detected despite extensive renal function testing (9,13). Complete exchange transfusions with adenine fortified blood in neonates had no detectable physical, biochemical or renal effects and up to five years later (13).

Consideration of an upper limit of adenine infusion dosage has heretofore relied upon the presumption that urinary DOA solubility was similar to that in water (=2 mg/l) (1). Recent studies, however, indicate that human urine displays a remarkable ability to maintain large quantities of DOA in stable, supersaturated concentrations both in vitro and in vivo (18).

Summary

In summary, extensive evidence supports the conclusion that 10-15 mg/kg of acutely infused adenine is not associated with serious toxicity. DOA crystalluria is to be expected, but does not imply renal damage. Assuming adequate renal function, 40-60 units of fresh CPD-II, acutely administered and totally available (i.e., not lost via hemorrhage), would appear to be safe for each twenty-four hour period.

TABLE I - ANIMAL STUDIES

Animal	Route	Dose	Toxicity
Mice (CFW, AKM)	IP*	Single	LD_{50} = 335 mg/kg (1)
Rat (Wisteri)	IP	Single	LD_{50} = 198 mg/kg (1)
Rat	Oral	Single	LD_{50} = 745 mg/kg (1)
Rat (normal)	IV**	50 mg/kg	No renal function changes or lesions (2)
Rat (normal)	IV	50 mg/kg X 14 days	No renal function changes or lesions (2)
Rat (dehydrated)	IV	50 mg/kg	No renal function changes or lesions (2)
Rat (unilaterally nephrectomized)	IV	25 mg/kg	No renal function changes or lesions (2)
Rat (normal)	IV	50 mg/kg	BUN elevated at 24 hours; returned to normal at 48 hours, renal crystal formation (2)
Dalmatian Dog	IV	25 mg/kg acutely	Well tolerated (2)
Dalmatian Dog	IV	10 mg/kg daily X 15 days	Well tolerated (2)
Mongrel Dog	IV	50 mg/kg acutely	Well tolerated (2)
Holtzman Rat	IV	50 mg/kg during 6-15th day of gestation	No teratologic effect (2)
N.Z. Rabbits	IV	50 mg/kg during 6-15th day of gestation	No teratologic effect (2)
Beagle Dogs	IV	10 mg/kg 5-6X/week X 3 weeks	Well tolerated (5)
Monkey (*Macaca irus*)	IV	10 mg/kg/day X 5 days	Well tolerated (7)

* IP = intraperitoneal
**IV = intravenous

TABLE II - HUMAN STUDIES

1. Case report: dose = 5000 mg/day orally x 6 1/2 days. Developed severe uremia. Complete recovery except BUN remained 32-38 mg% (3).

2. Case report: IV dose = 95 mg/kg over five days as ACD-adenine blood. Died on the seventh day of hemorrhagic shock; had impaired renal function and renal crystalline deposits were noted at autopsy (11).

3. Series: Five patients received 8.7 - 15.1 mg/kg as ACD-adenine blood; all died within 12 hours of infusion of complications of open-heart surgery. No 2,8-DOA crystals in kidney tissues at post mortem exam (11).

4. Series: 118 patients IV dose = 2 - 15 mg/kg as ACD-adenine blood. No changes in serum creatinine up to 8 days following infusion (12).

5. Series: 27 children. IV dose = up to 12 mg/kg as ACD-adenine blood. Five years post-transfusion revealed no abnormalities in physical exam, serum creatinine or pitressin test, nor any evidence of renal damage (13).

6. Case report: IV dose = 10 mg/kg + tracer C^{14} label. Plasma disappearance half-life 30 minutes. Six hour urine sample contained 15% of dose as adenine, 4% as 2,8-DOA and 3% as 8-hydroxyadenine (10).

7. Series: IV doses of 0, 5, 10 mg/kg. No changes in a wide range of renal function tests acutely or 8 days later. Tests included an assessment of glomerular function (creatinine clearance, protein excretion), proximal tubular function (amino acid and glucose excretion), and distal tubular function (maximal acidifying and concentrating ability). Plasma disappearance half-life 25 minutes. 4% of 10 mg/kg dose was recovered as DOA in the 24 hour urine collection. Crystals of 2,8-DOA appeared in early urines. Urine was possibly supersaturated with 2,8-DOA (up to 100 mg/l) (9).

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APPENDIX C
Analysis of Data

General

Data collected on the proposed forms will be transcribed directly to IBM cards. Documentation, data description and various statistical analyses will be performed using SPSS and BMD Programs implemented via remote terminal (LAIR) on the CDC 7600 (Lawrence Radiation Laboratories, Berkeley, CA).

Data reduction and summary statistics of the various demographic and measured data will be computed using standard parametric techniques (mean, standard deviation, standard error of the mean) (1). Where departures from normality render parametric description sufficiently inaccurate, non-parametric techniques may be used (median, percentiles) (2).

Phase 1A -- Whole Blood and Packed Red Cell Survival

A one-tailed test of the hypothesis that the mean CPD-II RBC survival is at least 70% (3) will be made using the Student's t-test. It is fully expected on the basis of previous work (4,5) that mean CPD-II RBC survival will exceed 70% for both whole blood and packed cells, in which case under the above stated hypothesis, no statistical test need be performed. However, in the event that mean CPD-II RBC survival is somewhat less than 70%, a minimum of 30 cases will be required to adequately test the null hypothesis that a mean survival of 63% (±10%) or lower is significantly less than 70% at the $p \leq .05$ level (power = .85) (6). Should a significant departure from normality render the t-test invalid, the Wilcoxin Sum test can be applied (2).

If a sufficient number of cases is collected, an alternative data analytic strategy is available. A "quality-control" approach would be a one-tailed test of the hypothesis that at least 75% of CPD-II units evidence survival of 70% or greater. The standard test of a sample proportion against a population proportion would apply in this case (1). From previous studies, the expectation is that greater than 75% of CPD-II units will have survivals greater than 70% (4,5). However, if less than 75% of CPD-II units meet this criterion, a minimum of 43 cases would be needed to establish that 60% or less is a statistically significant departure from the test criterion ($p \leq .05$, power = .60) (6).

The RBC survival from whole blood and packed cells will be pooled for the above tests unless both of the following conditions hold: (1) a one-tailed, two-sample t-test reveals that the mean survival of packed cells is significantly less than that for whole blood ($p \leq .05$) and (2) a one-tailed t-test reveals that the packed cell survival is significantly less than 70% ($p \leq .05$; if n = 15 and survival is 65% ± 10%, power = .60) (6).

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APPENDIX D - Methods

Definition of the technical aspects of the protocol requires that certain minimal guidelines for developing data points be established. Any technique utilized should be at least as good as those defined in this appendix. In general, kinetic enzymatic assays are preferable to colorimetric methods. The methods used by each investigator shall be supplied to the data repository agents.

A. Red Cell Hemoglobin

This shall be obtained by a cyanomethemoglobin technique.

B. Hematocrit

The microhematocrit technique, or Coulter "S" method, shall be used.

C. Plasma Hemoglobin

Utilization of the optical density of an aliquot of the plasma read at 540 nm and plotted against a standard curve of hemoglobin at 540 nm is acceptable.

D. Reticulocyte Count

Standard reticulocyte staining methods utilizing supravital stains shall be utilized with counting of at least 1,000 RBCs.

E. Plasma Sodium and Potassium

Flame photometry with internal lithium standard shall be utilized.

F. Glucose

While no widely-used method for determination of glucose levels is considered the premier approach, several different techniques produce acceptable results. These methods include: (1) The color change of alkaline ferricyanide when it reacts with glucose and is read at 450 nm [this technique is easily adaptable to the Autoanalyzer (1,2)]; (2) Utilization of hexokinase to phosphorylate glucose (3). The phosphorylated glucose is then converted to 6 phosphogluconic acid by glucose 6 phosphate dehydrogenase (G6PD), and the increase in optical density as NADP is converted to NADPH is read in a spectrophotometer or spectrofluorometer.

G. Adenosine 5'-Triphosphate (ATP)

ATP is utilized to phosphorylate either glucose (4) or 3-phosphoglyceric acid (5). The reaction product is then coupled through an enzyme reaction to reduce NADP (glucose Rx) or oxidize NADH (3 PGA Rx). The resultant change in optical density is read spectrophotometrically or spectrofluorometrically. Equally acceptable are techniques which utilize the firefly bioluminescence as an indicator of ATP levels.

H. 2,3 Diphosphoglyceric Acid (2,3-DPG)

The enzymatic methods based on either of the following principles are acceptable: Utilization of 2,3-DPG as a co-factor in the monophosphoglycerate mutase reaction or, alternatively, reactions in which 2,3-DPG is used as a substrate in the 1,3 diphosphoglyceric acid NADH linked reaction (6,7,8,9).

I. pH

Determination of the pH of the stored blood preferably shall be made anaerobically at 4°C, room temperature, or 37°C. If obtained at other than 4°C, the temperature at which the pH was determined shall be reported.

J. Erythrocyte Survival Studies

Acceptable techniques shall be those in which an aliquot of cells at the end of storage is labeled with Cr^{51}. The methods utilized are to conform to the recommendations of the International Committee for Standardization in Hematology (12). The dose of Cr^{51} should never exceed 1.5 μCi/kg body weight, and the aliqubt of cells tagged should be 30-50 ml. A preinfusion blood sample from the recipient for baseline counting is obtained. The labeled aliquot of cells is then injected into the recipient, and blood specimens are obtained every five minutes for the first 20 minutes, then at 30 and 60 minutes, and finally 24 hours postinfusion. The specimens should be obtained from a vein other than the one in which the original sample was injected. Solid heparin or EDTA should be the anticoagulant. Extrapolation of the Cr^{51} counts back to time zero will allow accurate determination of the blood volume. Alternatively, a double isotope technique in which I^{125} is used to label albumin and, thus, determine plasma volume at the time Cr^{51} cells are reinjected is acceptable (13). Counting of the specimens in a gamma

counter should be undertaken in lysed samples (Water, Sterox or Saponin).

K. Blood Urea Nitrogen

An autoanalyzer method which utilizes diacetyl monoxime or a related compound will be acceptable for analysis of this compound in recipients of banked blood.

L. Creatinine

Shall be determined by utilization of a technique which quantitates the formation of red pigment with alkaline creatinine picrate.

M. Urine Hb

Presence or absence of hemoglobin in urine will be determined by use of a "dipstick" ordinarily utilized in urine screening tests, accompanied by a positive control to insure reactivity of the stick.

N. Platelets will be enumerated by phase contrast microscopy (14).

O. Factor VIII shall be determined by a one-stage assay employing a congenitally-deficient substrate, with factor VIII activity known to be less than 1.0% and an activated partial thromboplastin reagent. No less than two dilutions shall be determined in duplicate (15,16).

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10. An outline of any phase or phases of the planned investigations.

Details of the planned investigations are given in the clinical protocol as shown on the following pages.

final Format as of Jan 11

PROLONGED MAINTENANCE OF 2,3-DPG IN LIQUID BLOOD STORAGE: USE OF AN INTERNAL CO_2 TRAP TO STABILIZE pH

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Prolonged maintenance of 2,3-DPG in liquid blood storage: Use of an internal CO_2 trap to stabilize pH

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A close relationship exists between the decrease in concentration of 2,3-diphosphoglycerate (2,3-DPG) and a fall in the pH of stored blood. Buffering the stored red cells with bicarbonate is one solution to the problem of maintaining pH during storage. The effectiveness of this buffer depends upon loss from the stored blood of carbonic acid in the form of CO_2. We describe a system in which the CO_2 is trapped in a small internal package which contains calcium hydroxide, or calcium hydroxide embedded in Silastic. A medium containing bicarbonate, adenine, glucose, phosphate and mannitol (BAGPM) is added after initial packing of the erythrocytes. With this approach, it has been possible to maintain 2,3-DPG at 92 percent of original, and ATP was approximately 62 percent of initial levels at the end of 42 days of storage if an internal Silastic bag containing calcium was used in bags agitated once weekly. More frequent agitation (five times weekly) produced acceptable maintenance of both 2,3-DPG (78 percent of original) and ATP (44 percent of original) after 42 days of storage when a Silastic block impregnated with calcium hydroxide was utilized to absorb CO_2.

The increase in hydrogen ion concentration occurring during conventional liquid storage of blood is well documented.[1] A close relationship exists between the pH of stored blood and the concentration of critical intermediates such as 2,3-diphosphoglycerate (2,3-DPG) and adenosine triphosphate (ATP).[2-5] Alkaline storage conditions promote preservation of 2,3-DPG but cause a decrease in ATP concentration.[3, 4] Conversely, more acidic conditions are associated with a loss of 2,3-DPG while maintaining more normal ATP concentrations.[3-5]

A buffer system in the blood storage medium might provide stabilization of pH during storage. However, most nontoxic buffers with a pK_a in the appropriate range for blood storage have proved to be unsatisfactory. Phosphate accelerates glycolysis, thus hastening the formation of lactate.[6, 7] Tris helps maintain a relatively constant pH but has led to a rapid decrease in ATP while maintaining 2,3-DPG concentrations.[8] This may be due to phosphorylation in red cells of Tris,[9] loading the erythrocyte with an osmotically active substance and possibly depriving it of ATP. Bicarbonate is a normal constituent of blood. It

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Reprint requests: Ernest Beutler, M.D., Department of Hematology, City of Hope Medical Center, 1500 E. Duarte Road, Duarte, Calif. 91010.

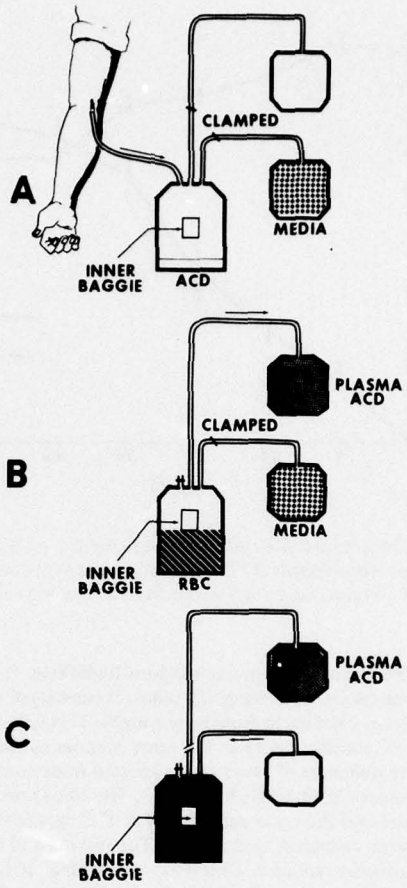


Fig. 1. Diagrammatic presentation of BAGPM storage method. *A*, The blood is drawn into a primary bag with ACD anticoagulant and the "inner baggie" containing $Ca(OH)_2$. The BAGPM medium is in a satellite bag. The combination is centrifuged, and the plasma is pressed into an empty satellite bag *(B)*. *C*, The BAGPM is then added to the packed cells from the satellite bag.

was originally used as an experimental buffer because, although its pK_a is quite low, very high concentrations of this nontoxic material could be employed.[10] Bicarbonate buffer was found to be especially effective when carbon dioxide produced from carbonic acid escaped from the plastic blood bag, much as CO_2 loss from the lung prevents acidosis.[11]

However, these earlier studies revealed the rate of CO_2 loss to be greatly dependent on the composition and size of the blood bag.[10] This report introduces an alternative method for removal of CO_2 with the use of an internal CO_2 trap.

Materials and methods

The CO_2 absorber, termed a "baggie," was inserted by opening the bottom of an empty 600 ml. transfer pack (Fenwal PL 130 plastic) and then resealing with a hot iron and autoclaving for 20 minutes at 121° C. The baggie* consisted of a 0.012 inch thick Silastic bag which was 80 mm. long by

*Generously supplied by Mr. Eldon Fritch, Dow Corning Co., Midland, Mich.

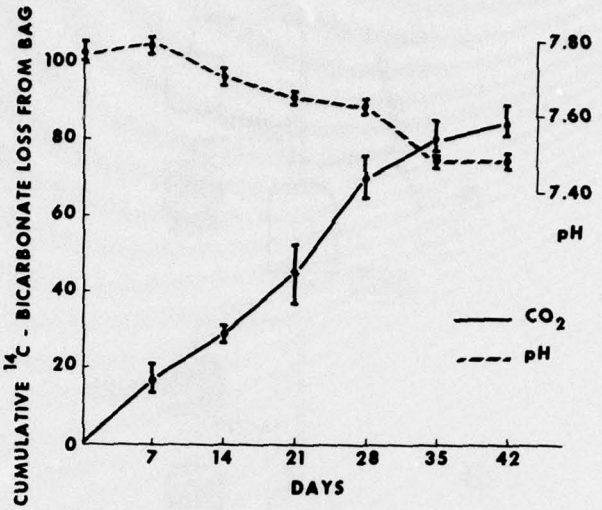


Fig. 2. The cumulative ^{14}C-bicarbonate loss per week in percent (——) and the change in pH (-----) are both plotted against days. The amount of ^{14}C-bicarbonate lost is about 84.5 percent by 42 days, and the pH has dropped from 7.75 on day zero to 7.49 on day 42. This reveals the buffering ability of the bicarbonate system.

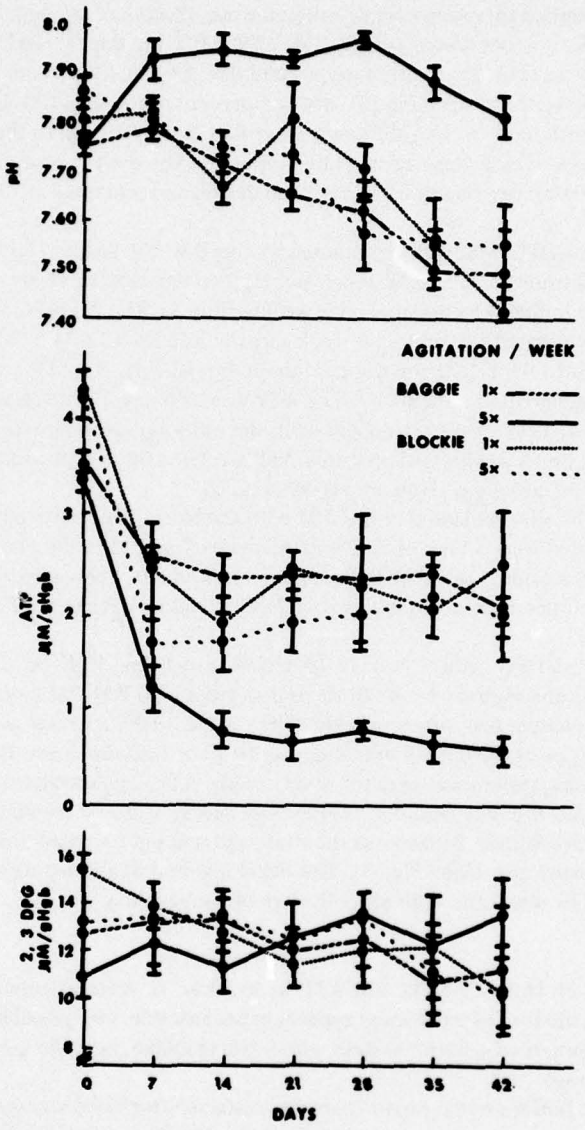
60 mm. wide and contained 6 gm. of granular calcium hydroxide. A further modification of this internal CO_2 absorber was employed in additional studies. It consists of a block of Silastic* 3.0 by 3.0 by 1 cm. containing 17 percent calcium hydroxide by weight. This CO_2 absorber, termed a "blockie," was inserted into the 600 ml. transfer packs in the same manner as the baggie.

Four hundred and fifty milliliters of blood were collected from normal healthy adult donors into 67.5 ml. of acid-citrate-dextrose (ACD) (NIH formula A). The blood was allowed to stand for 15 to 30 minutes at room temperature and then was centrifuged at 4° C. at 4,500 × g for 5 to 10 minutes in a Sorvall RC-3 centrifuge with a swinging bucket rotor. The plasma and buffy coat were both pressed off, and 200 ml. of preservative solution (BAGPM) containing 101.4 mM sodium bicarbonate ($NaHCO_3$), 14.3 mM sodium carbonate (Na_2CO_3), 1 mM bisodium phosphate (Na_2HPO_4), 1 mM adenine, 55 mM glucose, and 0.5 percent mannitol was added for each volume of plasma expressed.[7] After gentle but thorough mixing, the red cell suspension was aseptically transferred into the bag containing the baggie or blockie. A schematic drawing of how such a system might be reduced to practice is shown in Fig. 1. The red cells were then stored at 4° C. for 42 days and agitated for 1 minute, either once at the time of sampling or five times weekly, to ensure thorough mixing.

Erythrocyte ATP was quantitated by the hexokinase method,[12] and the concentration of 2,3-DPG was measured by a modification of the technique of Krimsky.[12] The pH was determined by a technique which allowed anaerobic measurements at 0 to 4° C.[13]

The loss of bicarbonate from the blood as CO_2 during storage with the baggie was measured by use of ^{14}C-bicarbonate added to the BAGPM media. The bags were placed in sealed dessicator jars, each containing a small beaker partially filled with dimethylbenzyl ammonium hydroxide (Hyamine), which because of its high pH, absorbs CO_2 quantitatively. At weekly intervals, the jars were opened, and the Hyamine was removed, counted, and replaced with fresh Hyamine. A 0.5 ml. sample of well-mixed, stored red cell suspension was removed weekly from the storage bag (at the time of blood collection for all other studies). The sample was then placed in a Warburg flask containing 2.5 ml. of distilled water in the bottom with 0.2 ml. of Hyamine in the center well and 0.5 ml. of 30 percent trichloracetic acid (TCA) in the side arm. The flask was sealed, and the TCA was tipped into the flask to convert all bicarbonate to CO_2. After 2.5 hours' incubation at 37° C. in a metabolic shaker, the Hyamine was removed and counted.

*Generously supplied by Mr. Eldon Fritch, Dow Corning Co., Midland, Mich.



METABOLIC PARAMETERS IN BAGPM STORED BLOOD

Fig. 3. The pH, ATP, and 2,3-DPG levels of the blood stored with the baggie or blockie are plotted for 42 days of storage. The units stored with the baggie were agitated once weekly (—·—·—) or five times per week (——). The units stored with the "blockie" were either agitated once weekly (.....) or five times per week (-----). The results reveal the critical drop in ATP which occurs when the pH rises during storage, i.e., units stored with baggie and agitated five times a week.

Results

A total of 6 units of blood were stored in BAGPM with the baggie and agitated once a week. The pH remained in a very narrow range for the 42 days of storage, beginning at 7.76 ± 0.1 (S.E.M.) on day zero, peaking at 7.79 ± 0.2 on day 7, and dropping to 7.49 ± 0.09 on day 42 (Fig. 2). By the forty second day, 84.5 ± 1.4 percent of the bicarbonate originally present was lost (Fig. 2). About 2 percent of the total CO_2 loss per week was found to be attributable to CO_2 diffusing externally from the bag. In the three units containing the baggie which were agitated five times weekly, the pH rose more abruptly from the 7.75 ± 0.01 on day zero to 7.97 ± 0.02 on day 28 and remained at 7.81 ± 0.04 on day 42 (Fig. 3).

Erythrocyte 2,3-DPG was well maintained in the BAGPM baggie storage medium. The levels dropped from 13.05 ± 0.78 μmol/gm. Hgb on day zero to 11.36 ± 3.32 μmol/gm. Hgb on day 42 in the units agitated once weekly (Fig. 3). The 2,3-DPG concentration of red cells in units agitated five times per week actually rose from 11.04 ± 0.78 μmol/gm. Hgb on day zero to 13.54 ± 1.35 μmol/gm. Hgb on day 42 (Fig. 3). ATP levels reflected markedly the difference in the pH milieu. The ATP was 3.59 ± 0.1 μmol/gm. Hgb on day zero and 2.05 ± 0.41 μmol/gm. Hgb on day 42 in the units agitated once weekly (Fig. 3). Units agitated five times weekly had an initial ATP level of 3.38 ± 0.04 μmol/gm. on day zero and 0.56 ± 0.12 μmol/gm. Hgb on day 42 (Fig. 3).

Four units of blood were stored in BAGPM with the blockie and agitated one time per week. The pH ranged from a high of 7.82 ± 0.02 on day 7 to 7.41 ± 0.03 on day 42 (Fig. 3). Seven units containing the BAGPM-blockie combination were agitated five times weekly, and these units maintained their pH at 7.86 ± 0.03 on day zero to 7.55 ± 0.08 on day 42 (Fig. 3).

Intracellular 2,3-DPG which was 13.16 ± 0.88 μmol/gm. Hgb on day zero was 10.22 ± 2.35 μmol/gm. Hgb on day 42 in the units stored with BAGPM-blockie combination that were agitated five times weekly (Fig. 3). 2,3-DPG, which was originally 15.3 ± 1.61 μmol/gm. at the time of drawing, was 10.13 ± 1.20 μmol/gm. Hgb on day 42 if the BAGPM-blockie system was agitated once weekly. ATP concentrations in the erythrocytes stored in the BAGPM medium with Silastic blocks were better maintained than the ATP levels of the Silastic baggies. In the units agitated once weekly, the day 42 ATP was 2.40 ± 0.64 μmol/gm. Hgb (Fig. 3). The blood agitated five times weekly had ATP levels of 1.88 ± 0.26 μmol/gm. Hgb after 42 days of storage (Fig. 3).

Discussion

Maintenance of both 2,3-DPG and ATP at as close to original concentrations as possible for long periods of liquid storage appears to be desirable. One possible approach to this goal is development of a buffer system which will stabilize the hydrogen ion concentration during storage.

A bicarbonate buffer system might seem unsatisfactory for blood storage because the pK_a of this system is approximately 6.6 at 0° C.[14] and offers relatively little buffering capacity in the pH range of 7.4 to 7.6. However, the loss of CO_2 from the blood allows this system to function in a highly efficient manner. The following reactions are involved:

$$\text{Glucose} \downarrow \quad H^+Lac^- + Na^+HCO_3^- \rightleftharpoons Na^+ Lac^- + H_2CO_3$$

$$H_2CO_3 \xrightleftharpoons{\text{carbonic anhydrase}} H_2O + CO_2$$

Previous studies have demonstrated that the surface-to-volume ratio is of critical

importance in determining the rate of CO_2 loss through plastic bags.[11] Satisfactory results were obtained only in small bags of 75 ml. capacity. Larger capacity bags, such as those utilized with a full unit of blood, were not satisfactory when the bag was made from polyvinylchloride plastic of 0.015 inch thickness. Other plastics including polyethylene, silicon rubber, and polyvinylchloride-coated silicon rubber were studied by us and have been found to permit either too much or too little permeation to CO_2, thus causing an unacceptably high or low pH. The development of an internal absorption system, consisting of a small Silastic bag containing calcium hydroxide or a Silastic block impregnated with calcium hydroxide, has been shown to be effective in maintaining pH, and therefore ATP and 2,3-DPG, at levels considered to be consistent with greater than 70 percent survival on reinfusion.[15] The loss of radioactive bicarbonate from the storage medium demonstrated that CO_2 is absorbed by the calcium hydroxide in the act of buffering.

The abrupt loss of ATP noted in the three units containing the baggie which were agitated five times per week reveals one of the problems associated with a storage system utilizing a diffusion gradient (of CO_2) to maintain pH. This difficulty appears to have been overcome by embedding the calcium hydroxide in a Silastic block and thereby apparently changing the rate of loss of CO_2 from the bag.

Although no in vivo erythrocyte survivals were undertaken in this study, it has been previously demonstrated that erythrocytes stored in artificial media containing bicarbonate have satisfactory survival on reinfusion through 6 weeks of storage.[10]

The authors gratefully acknowledge the technical help of Judy Metro and Sp-5 Victor Cheong.

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MAINTENANCE OF LOW SCREEN FILTRATION PRESSURE IN BLOOD STORED IN A NEW LIQUID MEDIUM: BAGPM

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Maintenance of low screen filtration pressure in blood stored in a new liquid medium: BAGPM

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Formation of microaggregates in blood stored in conventional media is reflected by rapidly rising screen filtration pressure (SFP). We show that the BAGPM (bicarbonate, adenine, glucose, phosphate, and mannitol) blood preservation system maintains SFP at near normal levels throughout the storage period of 42 days. Whenever the SFP had a tendency to rise in BAGPM blood, filtration through a routine in-line blood filter reverted SFP back to baseline levels. Blood from the same donors stored in citrate-phosphate-dextrose (CPD) adenine had a rapidly rising SFP by 7 to 14 days of storage. Filtration through the routine blood filter had no effect on the SFP of blood stored in CPD-adenine, CPD, or ACD. BAGPM not only maintains adequate levels of ATP and 2,3-diphosphoglyceric acid (2,3-DPG) during full 42 days of storage but also offers a unique system in which microparticulate material is prevented from forming, with maintenance of a low SFP, perhaps because of its low leukocyte, platelet, and fibrinogen content.

When blood is stored by conventional methods in liquid media, the pressure required to maintain flow through fine-mesh screen, the screen filtration pressure (SFP), rises rapidly as storage time increases.[1, 2] This increase in SFP is due to the formation of minute particles as the result of interaction between blood cells and possibly plasma fibrinogen.[3] When transfused, such particles, which are designated microaggregates, may result in harmful systemic effects especially to the lung and central nervous system.[4–6] Experimentally the deleterious effects of microaggregates have been prevented by their removal by filtration through specially designed very fine filters.[7–9] However, maintaining a flow rate through such filters is a major problem, which becomes most apparent when massive transfusion therapy is needed. It is under these very circumstances that the damage to the microcirculation by the microaggregates is likely to be greatest.[10–12]

BAGPM is used in a relatively newly developed blood storage system in which the plasma and buffy coat are removed and the red cells are preserved at 4° C. in a medium containing bicarbonate, adenine, glucose, phosphate, and mannitol. In previous investigations,[13, 14] we demonstrated that this system provided satisfactory levels of adenosine triphosphate (ATP) and 2,3-diphosphoglyceric acid (2,3-DPG) for at least 42 days. We now show that red cells collected in BAGPM maintain a low SFP throughout storage.

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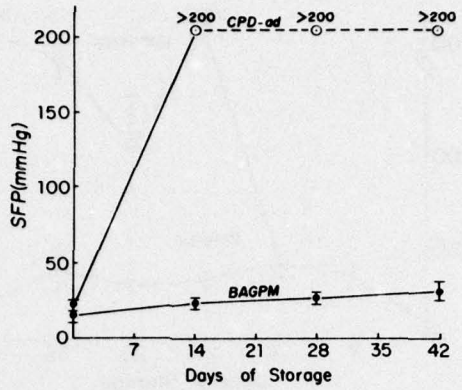

Fig. 1. Screen filtration pressure readings (mean ± S.E.) of blood stored in CPD-adenine (n = 6) and BAGPM (n = 12) during 42 days of storage at 4° C.

Table I. Comparison of SFP (mm. Hg) in blood preserved in CPD-adenine and BAGPM at various intervals

Storage medium	*Days of storage*			
	0	*14*	*28*	*42*
Mixed once a week:				
CPD-adenine (n = 6)	22.33 ± 4.46*	>200	>200	>200
BAGPM (n = 12)	14.75 ± 0.80	25.91 ± 3.57	32.25 ± 3.73	42.75 ± 6.13
Mixed 5 times a week:				
BAGPM (U)† (n = 4)	9.50 ± 0.90	42.50 ± 9.26	106 ± 24.70	>200
BAGPM (F)‡ (n = 4)	—	—	24.25 ± 1.93	20.25 ± 4.19

*Mean ± S.E.M.
†(U) = SFP of unfiltered samples.
‡(F) = SFP of same samples as in BAGPM (U) after filtration when readings were >50 mm. Hg.

Materials and methods

Healthy volunteers served as donors. Storage of 450 ml. units of blood in BAGPM medium was carried out as previously described.[14] Unless otherwise indicated, all of the units studied had an internal CO_2 trap consisting of either a "baggie" or a "blockie" containing calcium hydroxide, a system we describe elsewhere.[15] An additional 100 cc. of blood from six of the 12 volunteers was preserved in modified citrate-phosphate-dextrose (CPD)-adenine[16] at the same time for comparison of the SFP results. SFP readings of blood stored in ACD or CPD without adenine did not differ significantly from that of CPD-adenine blood when compared in 3 units of each. If the SFP reading was higher than 50 mm. Hg, each sample was filtered through a standard large-pore blood filter (McGaw Laboratories, Inc., Glendale, Calif.). Such filters are used routinely to remove fibrin clots in transfusion of blood and packed cells. Their pore size averages 164 μ in diameter, and they are inexpensive and offer little resistance to the rapid infusion of blood.

SFP's were measured with a Swank filtration apparatus[17] under identical conditions for BAGPM and CPD-adenine blood. Values presented represent the mean of the duplicate measurements of each sample at 37° C. (Table I).

Results

SFP of fresh BAGPM blood with hematocrit values of 40 to 50 percent was 14.75 ± 0.80 mm. Hg (mean ± S.E.M.) and that of CPD-adenine blood with hematocrits

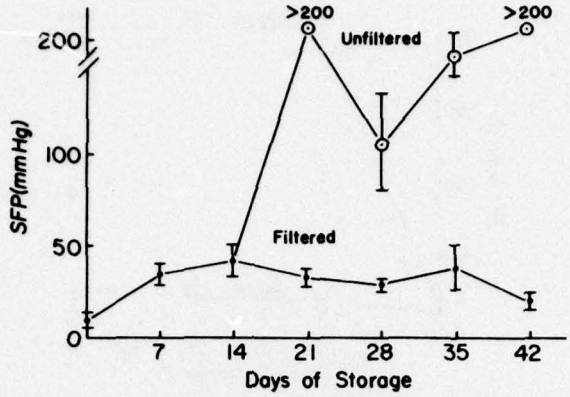

Fig. 2. Screen filtration pressure (mean ± S.E.) in 4 units of blood stored in BAGPM. Whenever SFP was higher than 50 mm. Hg, samples were filtered through a large-pore blood filter.

of 37 to 42 percent was 22.33 ± 4.46 mm. Hg. Striking divergence in the SFP of blood in the two storage media occurred as the time of storage progressed. By the seventh day of storage the SFP of CPD-adenine blood was over 200 mm. Hg, and by the fourteenth day it exceeded the 500 mm. Hg limits of the instrument even if filtered with the large-pore blood filter. Since the SFP readings of these systems were markedly different, subsequent readings of SFP were measured with instrument limits set at 0 to 200 mm. Hg range only, as shown in Fig. 1. The fact that the SFP of BAGPM blood units stored without the internal calcium hydroxide trap was essentially the same as of those with the baggie or blockie (results not shown) rules out $Ca(OH)_2$ as a contributor to this low SFP in BAGPM system. Blood stored in CPD or ACD without adenine had similar SFP readings when tested. In some BAGPM units stored with either baggie and blockie which were mixed five times a week, there was a tendency for SFP to rise by the twenty first day of storage when measured without passing through the conventional blood filter, but the SFP always reverted to the baseline readings when measured after filtering through the blood filter (Fig. 2). No such effect of the blood filter on CPD-adenine blood could be demonstrated: the SFP did not differ with or without filtration.

It was noted that at the time of the rise of SFP of unfiltered BAGPM blood, large pieces of a gelatinous coagulum could be observed in the suspended red cells. In control studies with blood collected in BAGPM without CO_2-absorbing blockies or baggies, the same coagulum was found. The effect of the standard blood filter on the SFP of BAGPM blood could readily be explained by removal of this material. When examined microscopically on Wright-stained smears or in wet preparation, this material appeared to have no definite structure. When dissolved in 6M urea, absorbance readings at 260 and 280 nm.[18] indicated a protein content of 0.07 mg./ml. and a nucleic acid content of 0.005 mg./ml. which were comparable with the protein and nucleic acid concentration of 0.09 mg./ml. and 0.005 mg./ml., respectively, when a similar quantity of a freshly prepared buffy coat was dissolved in 6M urea. This finding indicated that even though it appeared amorphous, the gelatinous material was derived largely from leukocytes and platelets; it could not have been formed primarily from plasma proteins, which would not have a ultraviolet (UV) absorbing peak at 260 nm.

In six BAGPM units we found that the white blood cell (WBC) count was about 65

percent of that in patients' original blood but only 10 percent of the platelets remained and the fibrinogen level was less than 48 mg./100ml. (the lower limit of our laboratory's measurement). Fibrinogen content in CPD-adenine blood was in the normal range (250 to 350 mg./100 ml.). Of the white cells, over 95 percent were granulocytes. One unit of freshly collected red cells suspended in BAGPM was filtered through a mixture of microcrystalline and α-cellulose, which has been shown to remove over 99 percent of the leukocytes and most of the platelets.[19] No gelatinous coagulum formed, and the SFP remained essentially unchanged throughout the storage period of 35 days.

Discussion

Microaggregates are potentially harmful to patients undergoing blood transfusion therapy.[4–6] They may result in partial occlusion of the pulmonary microvasculature, the first portion of the microcirculation to be traversed by such particles. The occurrence of unexplained pulmonary changes in massively transfused patients has lent support to this concept.[10–12] In cardiac-bypass surgery, microparticles may produce similar effects in the systemic microcirculation.[20]

The main contributing elements to these microaggregates appear to be platelets, leukocytes, and fibrinogen which interact in some way between the third to seventh day of storage of blood in CPD or ACD, at which time SFP starts rising.[3] Substantial reduction of the number of platelets and concentration of fibrinogen and only moderate reduction in numbers of WBC's by the initial process of centrifugation in the BAGPM system seem to play a major role in decreasing formation of microaggregates. The SFP remains in the normal range or rises only slightly in blood stored in BAGPM. Even when the SFP does increase, simple filtration through an ordinary in-line filter brings the SFP back to baseline levels (Fig. 2). Although the somewhat reduced leukocyte count of BAGPM blood and its greatly reduced platelet count and fibrinogen content seem to play a role in the failure of the SFP to rise during storage, it seems clear that this is by no means the only explanation. What seems to occur during storage of blood in BAGPM is the coalescence of the remaining leukocytes and platelets into large gelatinous aggregates which either remain in the blood bag or which are easily filtered out by coarse in-line transfusion filters. A similar gelatinous coagulum is also reported to occur in frozen red cells[21] as well as in stored platelets.[22] The normal microscopic appearance of WBC's is lost when they are examined within 24 hours of storage of BAGPM medium. The identity of the gelatinous material with aggregated leukocytes and platelets is suggested by its high nucleic acid content and is confirmed by its failure to form when leukocytes and platelets are removed by filtration through cellulose powder.

It is not certain why this aggregation occurs, but it may be due to the high pH and the low protein concentration of the system.

Other means of preventing the infusion of microaggregates are also known, but these are expensive and inconvenient. The primary approach to this problem has been the removal of these microparticles by the use of specially designed filters rather than prevention of formation of such material.[2, 23] However, some such filters are relatively ineffective, and all are expensive and impede the rapid flow of blood required for the treatment of patients with massive blood loss.[23] The diminished number of platelets and leukocytes in frozen red cell preparations also results in reduced microaggregate formation.[21]

Our studies demonstrate that in addition to the other advantages which appear to be inherent in the BAGPM system—salvage of labile formed elements and plasma fractions,

relatively low cost, excellent preservation of viability and oxygen transport characteristics, low levels of potassium and citrate, and possibly reduced inoculum of hepatitis viruses—BAGPM may provide a red cell preparation which is relatively free of microaggregates.

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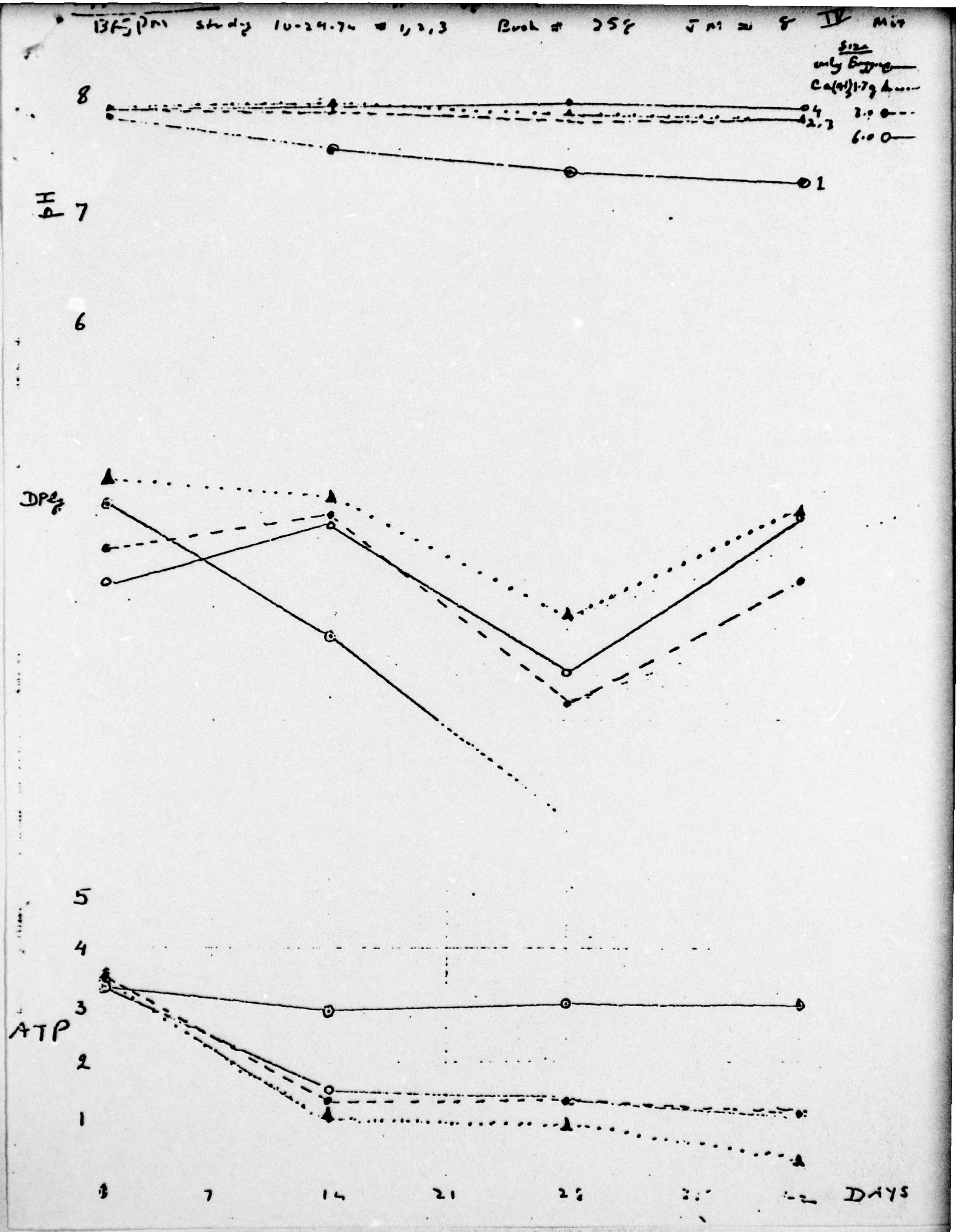
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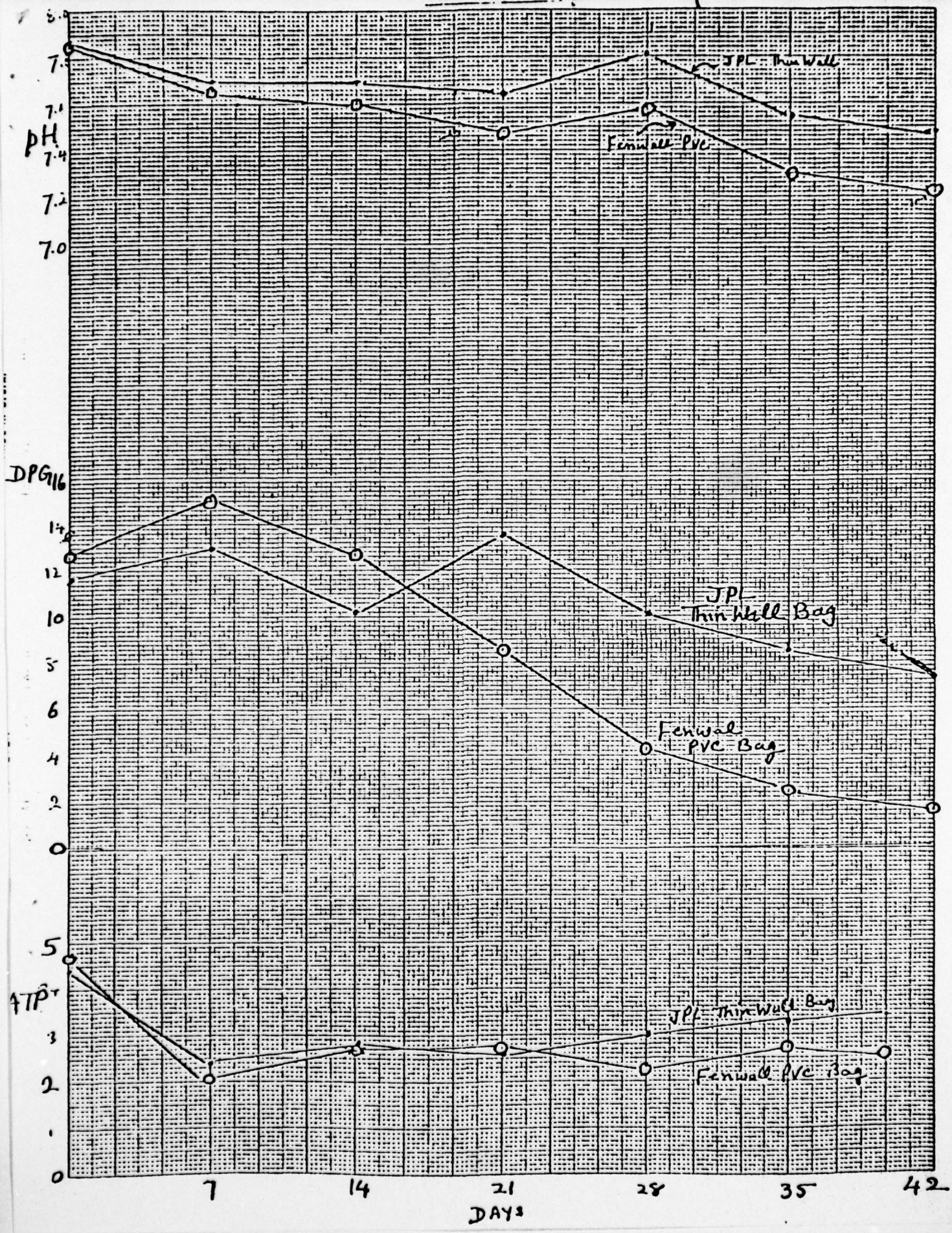
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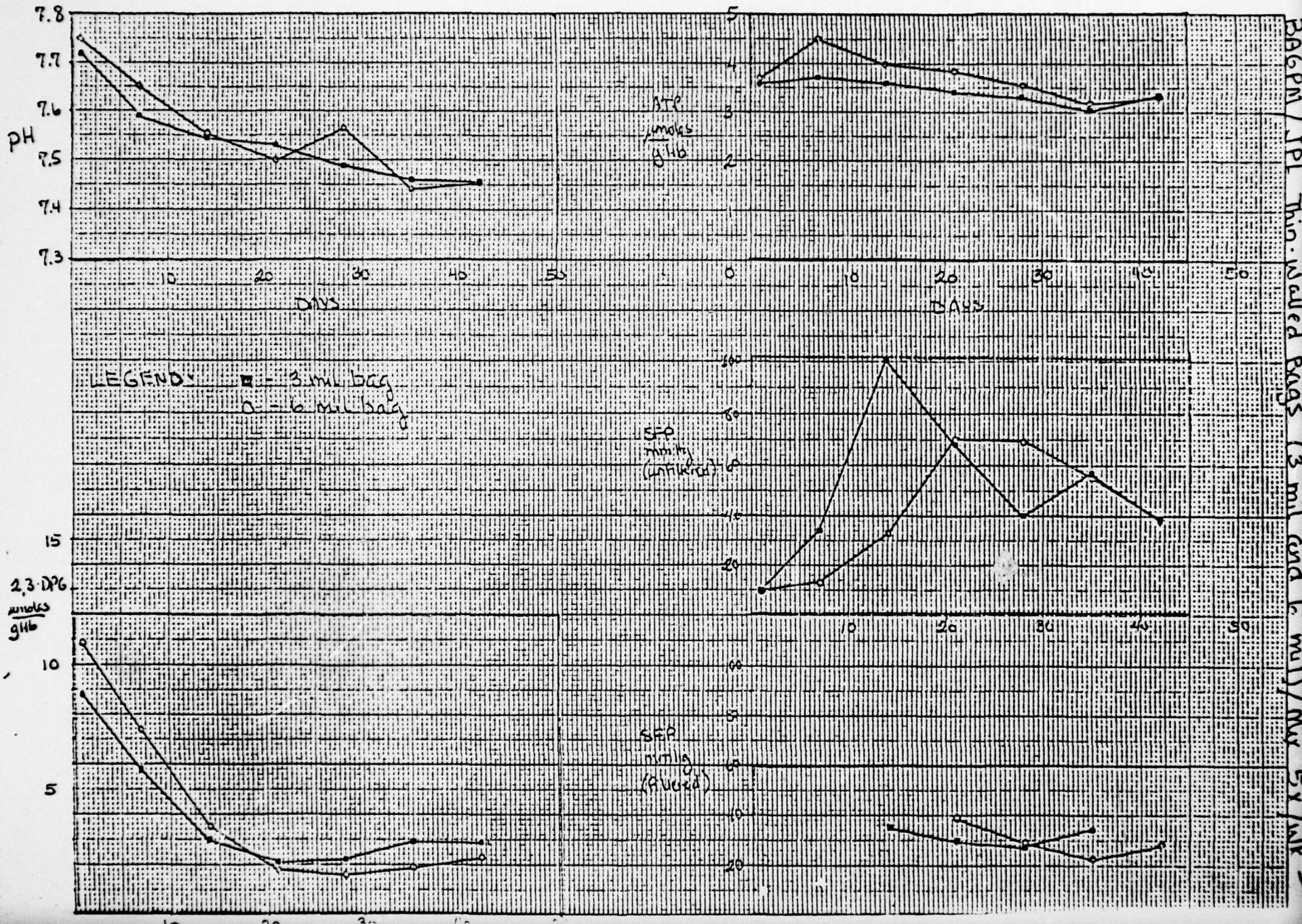

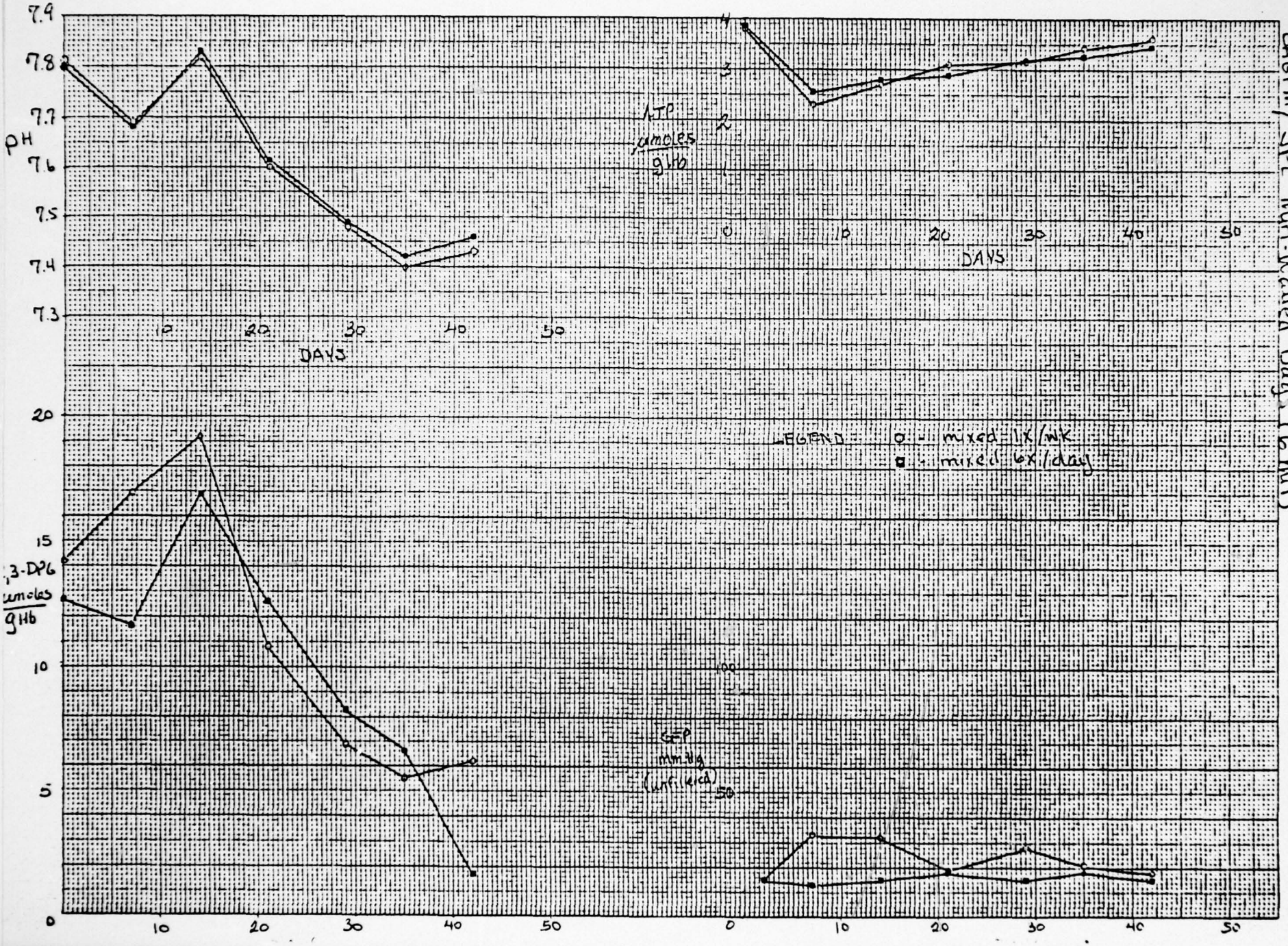

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This report reviews progress made under contract DADA 17-70-C-0078. Background information is given concerning the need for improved liquid storage, the relationship between liquid storage and frozen storage, and the potential importance of red cell 1,2-DPG in blood storage. Studies carried out under this contract, including the effect of mixing, the effect of various additives, such as dihydroxyacetone, the bicarbonate-adenine-glucose-phosphate-mannitol (BAGPM) preservative medium and the development of the CPD-II (CPD with added glucose and

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