# THE EFFECT OF STORAGE MEDIA ON CANINE BLOOD FOR TRANSFUSION



by

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# INTRODUCTION

## I. History

Successful transfusion of blood from one animal to another was achieved in the 17th Century using dogs.[93] This therapy was not used extensively in any species including man until the mysteries of blood coagulation and serologic incompatibility were solved. Earlier attempts to alleviate the clotting of blood included paraffin-lined containers, "direct" transfusions, cadaver blood and anticoagulants. The introduction of citrate to prevent coagulation and glucose to retard hemolysis and increase *in vivo* survival constituted the major advancement until the early 1940's.[63,65] To reduce carmelization of glucose during autoclaving, citric acid was used to acidify the solution.[44] Surprisingly, the lower pH further decreased red cell hemolysis. Extensive testing of the acid-citrate-dextrose (ACD) solution disclosed better survival in the recipient's circulation and no serious deliterious effects.

The concept of "blood banks" and the need associated with World War II stimulated research on blood preservation. As methodology improved and its significant role in therapy was realized, blood transfusion became a valuable procedure.

## II. Human Blood Storage

Because of its obvious medical significance, most of the research on blood storage used human erythrocytes. The major

drawback of stored blood was the relatively short shelf life. After three weeks of storage, the post-transfusion viability was reduced below the level (70%) recommended by the National Institutes of Health.

A. Post-Storage Viability

Erythrocyte viability was defined as the percent of transfused red blood cells present in the circulation 24 hours after transfusion as determined by radioactive chromate.[31,36] The methodology varied so widely in the past that comparison of data from different laboratories was difficult.[82] Recently, standard methods for radioisotope red cell survival studies were established to eliminate the interlaboratory differences.[39] An automated differential agglutination technique was advocated as a better method than the $Cr_{51}$ method; however, it has not been widely accepted.[85] Post-storage viability was apparently related to blood storage conditions. The life span of cells surviving 24 hours was normal unless shortened by immunological, chemical, mechanical, or toxic effects.[86] There was no correlation between 24-hour survival values and red cell life span.[85]

B. Studies Involving Erythrocyte Adenosine Triphosphate (ATP)

1. Storage Lesion- The loss of red cell viability in excess of normal aging was designated as the "storage lesion" of blood.[32] It was known for many years that the erythrocyte organic phosphates decreased and inorganic phosphates increased in stored blood.[44,45,46] Later, convincing evidence was

presented that the storage lesion was primarily related to the decreased red cell ATP levels.[26,51] Deterioration of the erythrocyte membrane was described as a function of this storage lesion.[50] In addition to losing their ability to undergo structural changes, the red cell membranes lost lipids, became less hydrophobic, and lost sulfhydryl groups. The rate of decrease for ATP was less in ACD solution than the citrate preservative used prior to 1943.[62]

Blood storage research was directed at alleviation of the storage lesion. The basic premise was that manipulations stabilizing erythrocyte ATP levels would elevate post-storage viability and thus increase the shelf life of stored blood.[75] The major thrust was to improve the preservation media by addition of chemicals to ACD solution.

2. Purine and Purine Nucleosides- Addition of the purine nucleosides (adenosine, inosine, and guanosine) extended the preservation of stored red cells.[29] The effect of the nucleosides was believed to be a source of phosphorylated ribose providing energy for the erythrocytes.[33] Although this reaction was well known, it alone could not explain the viability effects. Initially, inosine was selected as the most suitable of the nucleosides for red cell preservation because it was less toxic than adenosine and a better substrate than guanosine for nucleoside phosphorylase.[29]

The net synthesis of adenine nucleotide which occurred

after incubation of nucleotide-depleted red blood cells with adenosine, pointed out the importance of the purine moiety.[57] When attempts to duplicate the original viability studies were unsuccessful, the inosine purity was questioned.[43]

Subsequently it was confirmed that the purine bases, particularly adenine, were helpful in preserving stored blood. When adenine and inosine were incubated with blood previously stored for 8 to 10 weeks, the level of ATP increased and the altered erythrocyte appearance reverted to normal or near normal.[52] No change occurred when cells were incubated with either compound alone. Addition of inosine and adenine to ACD preservative at the beginning of storage maintained erythrocyte shape, osmotic resistance, ATP and post-transfusion viability. If added at the beginning of storage, adenine alone maintained viability and ATP for 5 weeks.[76] The effect of adenine was presumably mediated through net synthesis of ATP.

3. Phosphate- Phosphate exerted a beneficial effect on ATP levels of stored blood.[8] Although the exact mechanism for the phosphate effect was not clear, it effected hexokinase reaction by removing glucose-6-phosphate inhibition and stimulating phosphofructokinase and glyceraldehyde phosphate dehydrogenase activity.[23,49,53,64] The addition of phosphate and adenine to stored blood produced a profound elevation of erythrocytes ATP.[94] Under these conditions, after 50 days

storage, the ATP content was no longer the limiting factor in erythrocyte viability.

4. Pyruvate- Pyruvate was included in several media designed to increase ATP.[12,30,56,60] It was converted to lactate by lactate dehydrogenase with a concominant oxidation of reduced adenine dinucleotide. Increased nictonamide adenine dinucleotide concentration stimulated glyceraldehyde phosphate dehydrogenase.[60] The resulting increase in glycolytic rate increased the ATP levels.

5. Hydrogen Ion Concentration- The importance of preservative pH on ATP levels was demonstrated by several investigators.[6,7,14] The optimum pH of the ACD blood mixture was approximately 7.5 at 4° C. Mixing blood with ACD solution (pH 5.0 at 25° C.) resulted in a blood-media pH of approximately 7.0 at 25° C. When the blood ACD mixture was cooled to 4° C., the pH increased to 7.5. Higher pH values increased hexokinase activity which rapidly depleted red cell ATP.

C. Studies Involving 2,3-diphosphoglycerate (DPG)

1. DPG and Hemoglobin Interaction- The oxygen affinity of blood increased during storage in ACD solution.[92] The mechanisms involved in this change became clear only recently. Organic phosphates, especially DPG, combined with deoxyhemoglobin, shifting the oxygen dissociation curve to the right.[5,21] The DPG content of red cells fell rapidly during storage in ACD

solution.[4,10,20] DPG levels decreased to 50% of normal in three days, 75% in six days and 95% in ten days.[83] The fall in DPG levels was closely correlated with the increased oxygen affinity of stored blood.[17]

2. Clinical Significance- The clinical significance of transfusing stored blood with low DPG levels has been uncertain. A decrease in patient red cell DPG concentrations and $P_{50}$ values occurred immediately after transfusion with DPG-depleted red cells.[88,89] However, no significant effect on the patient's systemic oxygen consumption or cardiac index was detected. Red cell DPG was regenerated in vivo to one-half the normal value within 4 hours and then gradually increased for several days, eventually reaching normal levels.[11,90] Blood with low levels of DPG was thought to be important only in patients requiring massive transfusions.[11]

3. Effect of Chemical Additives- When the importance of DPG as a regulator of hemoglobin functions became known, subsequent research was directed toward the preservation and regeneration of both ATP and DPG concentration of stored red cells. Higher blood-preservative pH was beneficial to DPG but, detrimental to ATP levels.[12,24,25] Red cell mutase, which was responsible for the formation of DPG, was favored by a neutral pH, but phosphatase, which catalyzed its decomposition, was more active at a acid pH.[28] The opposing effects of pH made it difficult to maintain both ATP and DPG levels

in stored blood.

Pyruvate had a beneficial effect on DPG concentration in addition to the previously discussed effect on ATP formation.[60] Ascorbic acid and methylene blue also elevated DPG levels in stored human blood.[42,96]

Inosine catabolism yielded ribose-1-phosphate which entered the pentose phosphate pathway and was converted, in part, to DPG.[55] Inorganic phosphate acted as a substrate in this reaction.

D. Regeneration of Stored Blood.

The ATP and DPG levels of blood previously stored 3 to 4 weeks in ACD media were increased by the addition of chemical additives.[30,55,56] A solution containing phosphate, inosine, and pyruvate significantly increased both ATP and DPG; 37° C. for 3 to 4 hours was more effective than 1° C. for longer periods.[1,3,17,38,52,77,78,79,80,97]

Multiple additions of adenine or adenosine were more effective in the maintenance of organic phosphates than single additions.[81] The addition of adenine or inosine did not alter the rate of cell membrane deterioration as measured by total lipids, sulfhydryl and sialic acid concentration.[50]

The warming periods, which could permit bacterial growth, and the extra handling were major objections to the routine use of regeneration procedures. Toxic effects of nucleosides may occur because of a high uric acid level and

toxic products of the purines.[9]

E. Plasma Replacement

Another approach to prolonging storage life of blood was the use of artificial media without plasma.[95] Blood could be collected in either heparin or ACD; the plasma removed, and the cells suspended in 1 to 2 volumes of a solution containing adenine, phosphate, glucose, and sodium chloride. Prior to transfusion, the suspending media was discarded and the cells resuspended in either plasma or saline solution.

F. Storage Containers

Initially all blood collected for storage was placed in evacuated glass bottles. Subsequently, plastic storage bags gained popularity with blood banks. Increased viability of red cells was obtained with bags.[74]

One possible disadvantage of plastic bags was the migration of the phthalate plasticizer into the stored blood.[40] Although the chemical has been identified in human tissue, its toxicity has not been determined.

G. Temperature

Routinely, blood has been stored at $4^{\circ}$ C. Storing blood at $10^{\circ}$ C. did not decrease post-storage viability. Warming blood to $22^{\circ}$ C. for 16 hours was not harmful; however, after 24 hours storage at this temperature there was significant hemolysis and loss of post-storage viability. Mechanical

agitation for one hour per week did not apparently damage the cells.[71] Mixing blood stored in CPD bags 5 times per week significantly increased red cell ATP levels and viability, and reduced plasma hemoglobin concentrations. These effects were not present in blood stored in ACD bags.[27]

H. Current Blood Banking Practices

Despite the obvious advantages found for the various additives, only three preservative media have been used clinically: ACD, ACD-adenine in Sweden and citrate-phosphate-dextrose (CPD) solution in the United States.[2] CPD (developed by Gibson et al.[35]) differed from ACD solution in several respects:

1) CPD contained less citric acid and included sodium phosphate;
2) the pH of CPD (5.60) was higher than ACD (5.00);
3) it was isotonic to human red cells, whereas ACD was hypotonic;
4) CPD-plasma required only one-third as much base as ACD-plasma to bring the pH within normal values.[34]

There have been conflicting reports regarding the ability of CPD to maintain red cell viability for 4 weeks of storage rather than the three week limit of ACD.[15,26,35,54,70,91] During the first two weeks of storage, DPG levels in CPD were better maintained than in ACD.[19] Because of the higher DPG levels, transfused blood delivered more oxygen to the

tissues.[24,69] The elevation of erythrocytic DPG concentration was primarily due to the higher media pH rather than the presence of phosphate ions.[68]

## III. Canine Blood Storage

The use of blood transfusions in dogs followed the development of human blood banks in the post-World War II era. At the annual meeting of the American Veterinary Medical Association in Miami Beach, the South Florida Veterinary Medical Association demonstrated a transfusion technique using dogs.[47] In addition, a physician (Dr. J. Griffitts) of the Dade County Blood Bank discussed human blood banking practices.[37]

Research in the intervening years has included blood incompatibility studies, selection of donor animals and method of administration to the recipient. Only one (Group A) of the erythrocyte antigens was important in producing post-transfusion complications.[84,87] Serologic reactions were minimized by using donors with A-negative type blood. Preferentially blood was transfused intravenously into recipients, but it can be given intraperitoneally or intramedullary, but not intramuscularly.[22,58,87] Similar to human blood, the canine's oxyhemoglobin dissociation curve was shown to be regulated by DPG.[16]

### A. Blood Storage

One of the most controversial issues in the use of canine blood has been the length of storage; yet, very little

research was directed at this problem. The maximum time reported for blood storage ranged from 5 days to 6 weeks depending on the source of information. A maximum of five days was advocated by Dr. Bild[13] based on a conversation with a professional technician from a human blood-bank laboratory. Some clinicals advocated 21 days based on studies using human blood. More recently Owen and Holmes[59] recommended 6 weeks based on the life span of viabile erythrocytes after storage but other investigators indicated that the life of transfused red cell drops off rapidly after 15 days of storage.[87]

Post-storage viability decreased in stored canine blood.[72] After three weeks at 4° C. less than 70% of the cells were viable. Similar decreases occurred with stored human cells. Storage at higher temperatures showed progressively lower survival rates. Serious and fatal reactions were expected to occur when transfused blood had been stored for 14 days at 20° C. There were no differences in survival of arterial and venous blood or infusion to dogs with or without spleens.

Hematocrit, plasma sodium and blood glucose showed little alteration during storage.[59] Plasma hemoglobin levels gradually increased during the first 4 weeks of storage and then there was an abrupt increase. The pH values progressively declined during the first 4 weeks and then gradually increased through the tenth week. Plasma potassium values increased until the third week then remained constant.

B. Use of Dogs as Human Models

Dogs were used to study the toxicity of ACD solution.[41] Rapid infusion of ACD in large amounts (10 ml/kg body weight) was lethal. To the average human this amound of ACD would be contained in 10 pints of blood; however, rapid infusion with 14 pints with no adverse effects was reported.[73]

Dogs were used for studying techniques of storing blood at ultra low temperatures.[66,67] Frozen canine blood was used successfully as an effective therapeutic treatment for dogs in hemorrhagic shock. The post-storage viability (24 hours) of frozen blood was slightly lower (60-81%) than normal (84-89%). Freezing human blood preserved normal ATP levels but viability decreased because of mechanical damage; and, although the DPG concentration fell, there was little change in the oxygen dissociation curve of hemoglobin.[48]

C. Erythrocyte Metabolism

Peculiarities in canine erythrocyte metabolism must be considered when developing blood storage media for dog blood. Canine red blood cells contained less ATP and produced lactate at a lower rate than human erythrocytes.[18] The hydrogen ion concentration had less effect on the glycolytic rate in the canine than the human. Purine nucleoside phosphorylase activity was absent in the canine red cell and thus, incubation with inosine, pyruvate, and inorganic phosphate at 37° C. did not restore DPG concentrations in stored blood.[55] When canine erythrocytes were stored at room temperature, the DPG concen-

tration decreased at 72-96 hours. DPG in human cells decreased in 24-36 hours. Erythrocyte ATPase activity was very low in canine erythrocytes, and the sodium and potassium ion concentrations of red cells were in equilibrium with plasma.[61]

## IV. Rationale for Dissertation

The purpose of this research was three-fold. First, it was determined if *in vitro* biochemical tests were effective in predicting *in vivo*, post-infusion viability and functional capacity of stored canine red blood cells. Second, commonly used blood storage media and containers were evaluated for efficacy in preserving canine blood; also, a limit of duration for storage was established. Third, chemical additives were added to standard preservative media to increase the shelf life of canine whole blood.

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# PAPER 1. RELATIONSHIP OF *IN VITRO* BIOCHEMICAL PARAMETERS TO VIABILITY AND FUNCTION OF CANINE BLOOD STORED AT 4° C.

## SUMMARY

Adenosine triphosphate (ATP), 2,3-diphosphoglycerate (DPG), blood-preservative pH, and 24-hour post-infusion erythrocyte viability were determined in canine blood stored in acid citrate-dextrose (ACD) solution. These decreased significantly ($P<0.01$) during 6 weeks of storage. Canine blood maintained higher DPG levels than reported for human blood which indicates greater functional capacity to deliver oxygen to tissues. Post-infusion erythrocyte viability was significantly ($P<0.01$ correlated to DPG, ATP, and pH; therefore, these *in vitro* tests may be used to predict *in vivo* survival. Canine blood preserved in ACD solution should be stored for no more than 3 weeks.

## INTRODUCTION

Transfusion of stored canine blood is a frequently used therapeutic measure in veterinary practice. However, 2 general assumptions are made about the blood: first, that adequate numbers of red blood cells survive the collection, storage, and transfusion; and second, that these cells function adequately in the distribution of oxygen to body tissues.

The viability of human red cells is the limiting factor in the shelf life of stored whole blood. National Institutes of Health (N.I.H.) recommends that at least 70% of the transfused cells be viable 24 hours after transfusion; therefore, the storage of ACD preserved human blood is limited to 21 days.

This viability is highly correlated (r=0.95) with red cell content of ATP.[4,12,16,23]

The oxygen delivering ability of blood in the canine, human, and some other species is reported to be a function of the red blood cells' content of the organic phosphate, DPG.[7] This compound combines with deoxyhemoglobin to shift the oxyhemoglobin dissociation curve to the right and thus enables the hemoglobin to more easily release oxygen to the tissues.[1,10] The ability of red blood cells to increase their content of DPG is a sensitive, physiologic response to hypoxic stimuli. Elevated DPG levels can be found in such clinical situations as cardiovascular and respiratory insufficiencies and anemias; also, this response occurs as an adaptation to high altitude and strenuous muscular exercise.[8] There are species differences in the amounts of DPG present within the red cells. Man, dog, horse, rabbit, guinea pig and rat hemoglobin are strongly reactive with DPG and contain substantial quantities of DPG; whereas, sheep, goat, cow and cat hemoglobin are weakly reactive and have low concentrations of DPG.[7]

The ATP and DPG content of human red blood cells are known to decrease over a period of storage with an accompanying increase in affinity of hemoglobin for oxygen.[9,20,26] Furthermore, changes in pH of the blood-preservative mixture directly influence levels of the above mentioned chemicals.[3,5] DPG content of stored human blood can be increased by the addition of ascorbic acid to the preservative media.[27]

It was our purpose to follow the ATP, DPG, pH and viability of stored canine blood and attempt to establish the presence of relationships between the in vitro chemical parameters and in vivo viability and functional capacity.

## MATERIALS AND METHODS

Commercially prepared ACD solution "B"[a] was aseptically infused into 300 ml. capacity plastic bags.[b] Ascorbic acid solution was sterilized by passage through a 0.2 micron membrane filter[c] and then injected into 2 of the ACD bags giving a final concentration of of 80 mg./100 ml. of blood-preservative mixture. Blood (160 ml.) from each of 6 anesthetized dogs, weighing 14-23 kgs., was collected by jugular venipuncture. It was immediately chilled, then stored at 4° C. for 6 weeks.

Approximately 2 hours after collection, and thereafter at weekly intervals, the blood was mixed by gentle rotation and 22 ml. withdrawn through the plastic tubing of the bags. A 2 ml. aliquot was used for the in vitro measurements and the remainder for the viability studies.

ATP and DPG were measured by standard methods[2] using chemical reagents obtained from Sigma Chemical Company, St.

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[a]Abbott Laboratories, North Chicago, Illinois
[b]Transpak,R Abbott Laboratories, North Chicago, Illinois
[c]Metricel,R Gelman Instrument Company, Ann Arbor, Michigan

Louis, Missouri. Determinations of the blood-preservative pH were done anaerobically at the storage temperature, using a micro-blood sampling unit.[d] The pH meter was also calibrated at 4° C.

Erythrocyte survival studies were performed using the methods described by the International Committee for Standardization of Hematology.[14] Autologous transfusions of red cells tagged with $Cr^{51}$,[e] were carried out at weekly intervals, beginning 4 hours after the initial collection of the blood. The 24-hour viability percentages were calculated using the radioactivity of the 10 minute post-infusion blood sample as the 100% level and comparing it to the 24-hour post-infusion radioactivity. It was necessary to correct the measurements for chromium elution from red cells at the reported rate of 1-2% of the previous amount per day.[22] In addition, the counts were corrected for the radioactivity present from the previous week's experiment as measured by a pre-infusion blood sample.

Experimental data were anlyzed by analysis of variance with the 4 ACD solution controls versus the 2 ascorbic acid additive solutions as the main factors. The effect of time was also determined. The *in vitro* parameters were correlated to the *in vivo* viability using a multiple regression method.

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[d] Micro Blood Assembly, Beckman Instruments, Inc., Fullerton, California

[e] Radioactive sodium chromate, Amersham/Searle, Arlington Heights, Illinois

## RESULTS AND DISCUSSION

Red cell DPG, ATP, 24-hour viability, and blood-preservative pH decreased significantly ($P < 0.01$) over the 6 weeks of storage (Figs. 1-4). The decrease in viability of stored canine blood at 4° C. has been previously reported.[17,21] The addition of ascorbic acid did not produce a statistically significant difference from the controls.

It is interesting to compare our canine values with representative human values at the critical periods of storage. The most contrasting differences are found in the DPG levels (Fig. 1). Larger amounts of DPG are present in the canine blood than in human blood, especially at the end of the recommended 3 week storage limit. The ATP and viability differences are of a lesser magnitude, although they do appear to indicate higher values in the canine blood for comparable time periods (Figs. 2 and 4).

The very low amounts of DPG present in stored human blood after only 10 days of storage is of uncertain significance. Clinical situations calling for massive transfusions require near normal DPG concentrations to prevent a decrease in tissue oxygenation.[24] The decreased DPG in the blood, however, can be partially regenerated within 4 hours after transfusion and it continues to increase to normal levels within days.[6,25] Canine blood, on the other hand, maintained DPG content considerably higher for 2-4 weeks of storage. Therefore, the functional

aspects of transfusing canine blood may not be as critical as they are in man.

DPG, ATP, and blood-preservative pH decreased together. The relationship of *in vitro* measurements to *in vivo* viability was as follows: DPG level was the best predictor of 24-hour viability ($r=0.66$, $P<0.01$). To a lesser extent, viability could be predicted by ATP ($r=0.55$, $P<0.01$) and pH ($r=0.40$, $P<0.01$). In addition, ATP and DPG were closely correlated ($r=0.71$, $P<0.01$) and pH was significantly correlated to ATP and DPG ($r=0.61$ and $r=0.58$, $P<0.01$). The correlation of ATP and DPG to pH is the same as that described for human blood.[3,5,11]

Although the biomechanism of this relationship remains to be elucidated, the high correlation of DPG to viability in the canine should aid in predicting viability. DPG is a high energy glycolytic intermediate and thus may serve as a general indicator of the state of cellular metabolism. Possibly, the factors which were important for the viability of canine red cells are being preserved at the same time as DPG.

There are several reasons why ATP may not be the most important indicator of viability in the dog. In man, ATP is critical for maintenance of red cell shape and the $Na^+$-$K^+$ pump. On the other hand, this relationship is not found in the dog as the red cell content of these ions is at equilibrium with the plasma.[18] Also, the ATPase activity in dog red cells is practically absent.[19] Finally, it should be noted that the

normal canine red cell ATP concentration (1.80 micromoles/ Gm. hemoglobin, in our laboratory) is lower than that of humans (4.05 micromoles/Gm. hemoglobin[2]).

Owen and Holmes[17] have reported the maintenance of normal red cell half-life of canine blood stored in ACD for 4 weeks. Plasma hemoglobin concentrations remained low for this period. They further stated that after 1 week of storage, the blood should be transfused slowly and in small volumes to correct for a postulated low DPG content. Based on our data, this latter hypothesis does not appear completely valid. The relatively high concentration of DPG in canine blood stored 3-4 weeks would indicate a functional ability of the cells to transport oxygen to the tissues and possibly, a shorter time necessary for in vivo regeneration.

Moreover, if veterinarians wish to accept the same criteria of efficiency for stored blood that is widely used in human medicine, a 3 week storage limit should also apply to canine blood stored in ACD solution. Although clinically observable adverse effects may not be detected when blood is used after 4-6 weeks of storage, it is more reasonable to seek relatively high viability and good function in the stored blood. Therefore, blood stored less than 3 weeks would be more effective in correcting the clinical indication which prompted the transfusion therapy.

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Fig. 1- DPG concentrations in canine blood stored in ACD solution [standard deviation of a mean (S.D.)=1.21] and ACD-ascorbic acid (S.D.=1.71); DPG concentrations in human blood stored in ACD solution.[15]

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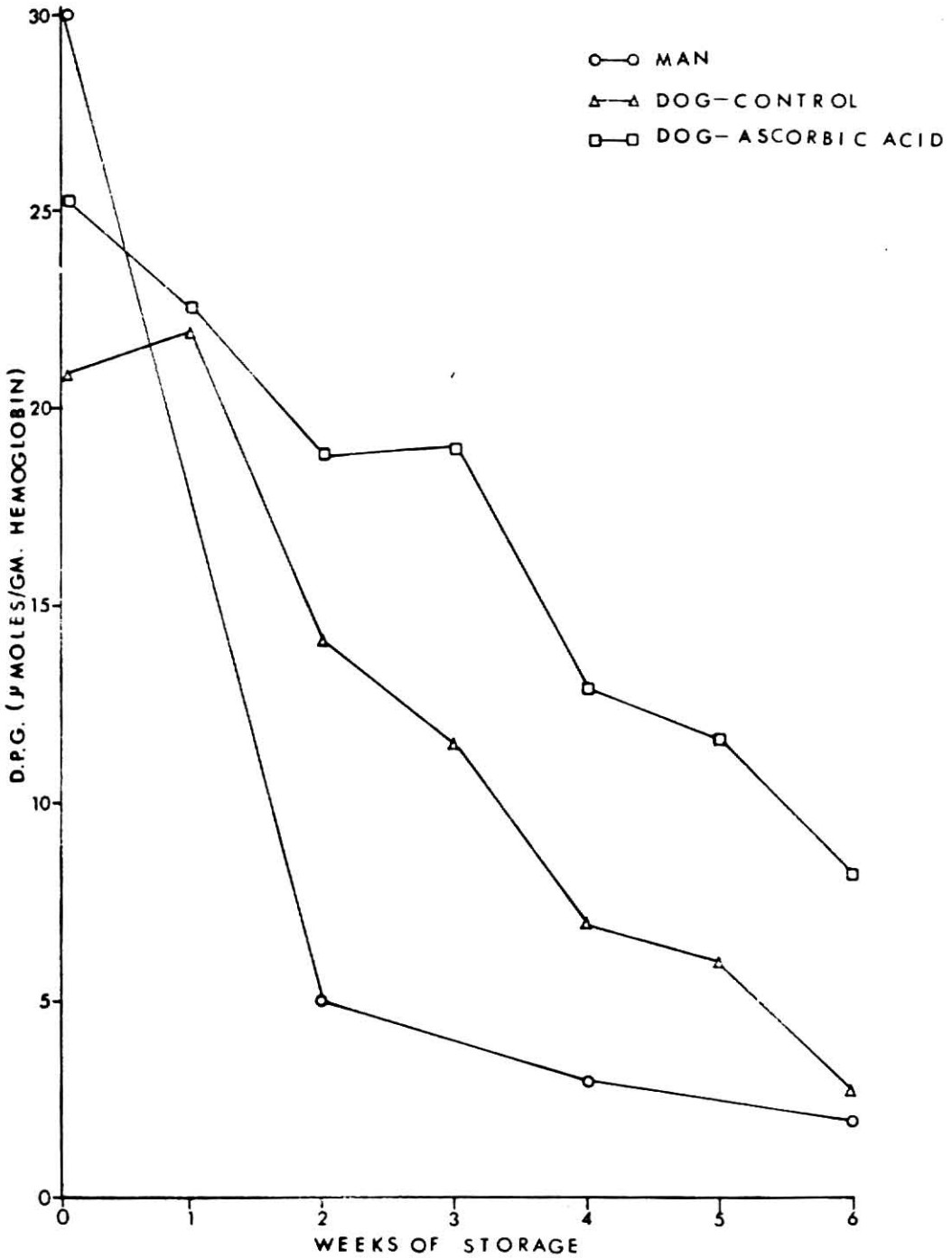

MAN
DOG–CONTROL
DOG–ASCORBIC ACID
D.P.G. (μ MOLES/GM. HEMOGLOBIN)
30
25
20
15
10
5
0
0
1
2
3
4
5
6
WEEKS OF STORAGE

Fig. 2- ATP concentrations in canine blood stored in ACD solution (S.D.=0.14) and ACD-ascorbic acid (S.D.=0.20); ATP concentrations in human blood stored in ACD solution.[15]

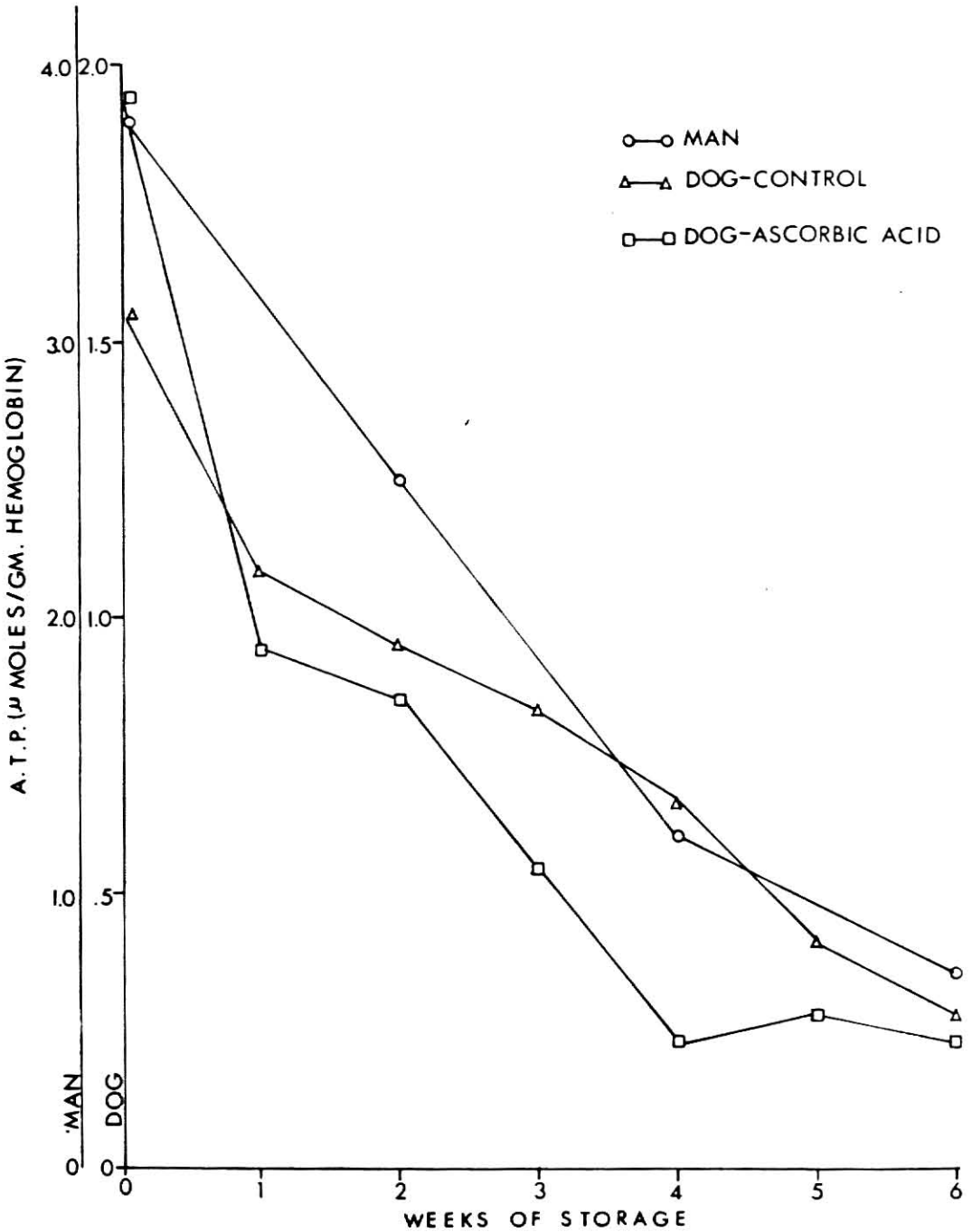

MAN
DOG-CONTROL
DOG-ASCORBIC ACID
A. T. P. (μ MOLES/GM. HEMOGLOBIN)
MAN
DOG
4.0
3.0
2.0
1.0
0
2.0
1.5
1.0
.5
0
0
1
2
3
4
5
6
WEEKS OF STORAGE

Fig. 3- pH levels of canine blood-ACD mixture (S.D.=0.02) and blood-ACD-ascorbic acid (S.D.=0.03).

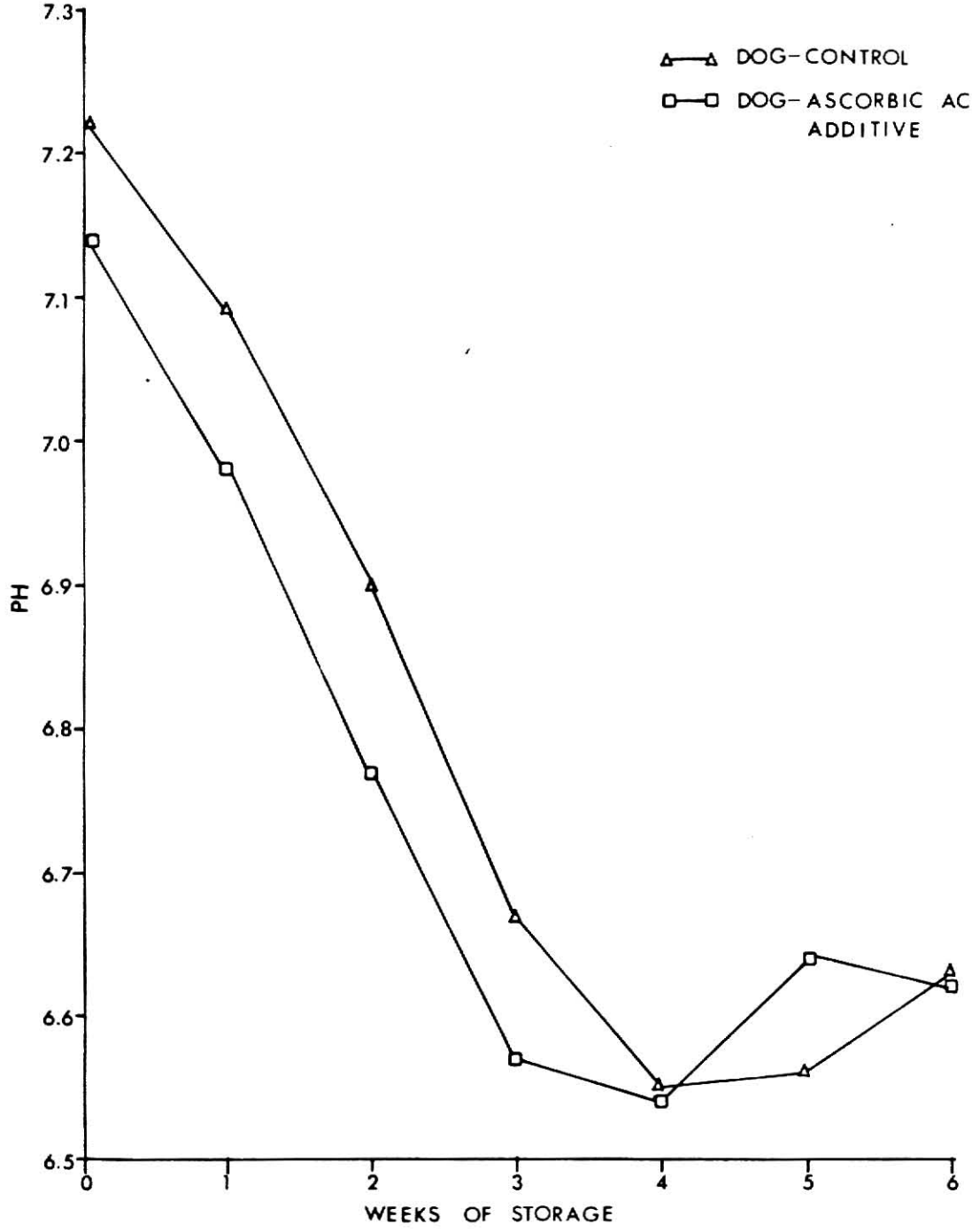
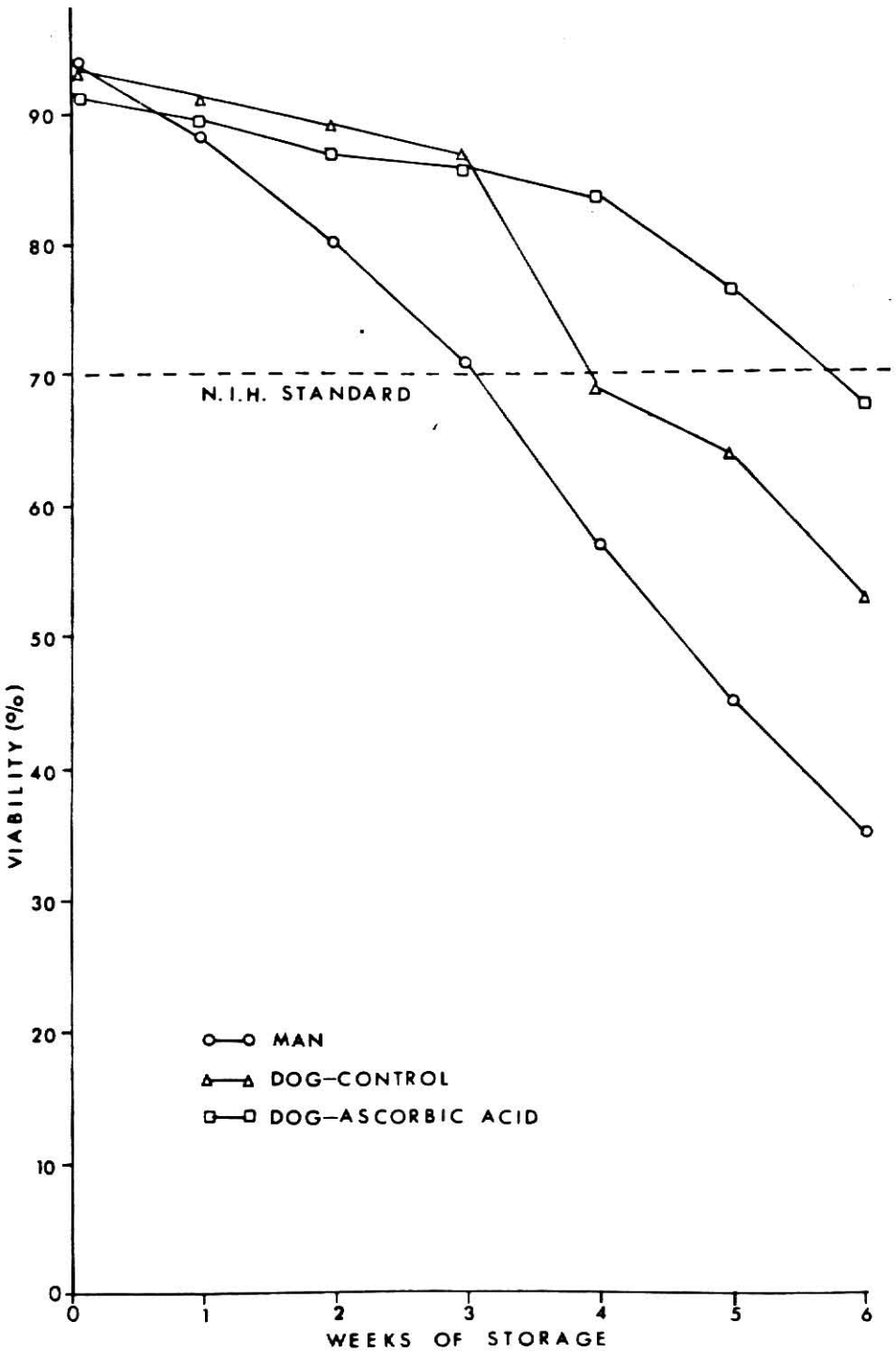

DOG-CONTROL
DOG-ASCORBIC ACID ADDITIVE
PH
7.3
7.2
7.1
7.0
6.9
6.8
6.7
6.6
6.5
0
1
2
3
4
5
6
WEEKS OF STORAGE

Fig. 4- Viability of canine blood stored in ACD solution (S.D.=6.4) and ACD-ascorbic acid (S.D.=9.1); viability of human blood in ACD solution.[13] The N.I.H. minimum standard for viability is indicated at the 70% viability level.


VIABILITY (%)
90
80
70
60
50
40
30
20
10
0
N.I.H. STANDARD
MAN
DOG–CONTROL
DOG–ASCORBIC ACID
0
1
2
3
4
5
6
WEEKS OF STORAGE

# PAPER 2. RELATIVE EFFICACY OF COMMONLY USED STORAGE MEDIA AND CONTAINERS IN PRESERVATION OF CANINE WHOLE BLOOD AT $4^{\circ}$ C.

## SUMMARY

The efficacy of acid-citrate-dextrose (ACD) and citrate-phosphate-dextrose (CPD) solutions in preserving canine whole blood for 8 weeks was determined by means of *in vitro* biochemical tests. CPD solution maintained 2,3-diphosphoglycerate (DPG) and pH closer to *in vivo* levels. Also studied was the effect of storing the blood in vacuum bottles and plastic bags. It is recommended that canine blood be collected and stored in CPD solution contained in plastic bags.


## INTRODUCTION

The storage of blood for transfusion has become a common procedure since the development of the first effective preservative solution, ACD, in 1943.[10] An extensive amount of work has been done in an attempt to develop media which would better maintain the viability and functional capacity of the blood. One such preservative, CPD, has gained wide usage today.[7] Additionally, polyvinyl chloride bags are replacing vacuum bottles for the collection, processing, and storage of blood.

The ability to predict good *in vivo* performance in man and dog has been dhown to be related to parameters which can be measured *in vitro*; these are red blood cell DPG, adenosine triphosphate (ATP), and the pH of the blood-preservative mixture.[3,5,11]

A 2 ml. aliquot from each container was aseptically removed with syringe and needle. The first samples were processed at 1 day of storage, with subsequent samples at 4, 7, 14, 21, 28, 35, 44, and 56 days. ATP and DPG were measured by standard methods[1] using chemical reagents obtained from Sigma Chemical Company, St. Louis, Missouri. Blood preservative pH was determined anaerobically. The pH determinations at 7 days storage and thereafter, were measured at 4° C. using a micro-blood unit.[d] The pH meter was also calibrated at this temperature. Due to technical problems, the pH of the samples at 1 and 4 days were determined at 40° C. using a different micro-sampling instrument,[e] then corrected to 4° C.[8]

The data were studied using analysis of variance with the 3 storage forms as the main factors. The effect of weeks was also determined. Correlations of the 3 measurements were performed using all 120 blood samples.

## RESULTS

Significant differences were found between the 3 storage methods tested. The CPD solution maintained DPG at a higher concentration ($P < 0.01$) than the 2 ACD solutions which were not different from each other (Fig. 1). Also, significantly

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[d] Micro Blood Assembly, Beckman Instruments, Inc., Fullerton, California

[e] pH/Gas Analizer[R] Instrumentation Laboratories, Boston, Mass.

different ($P<0.01$) pH levels were found between each of the 3 treatments (Fig. 2). The pH of the CPD-blood mixture was closest to physiologic, followed by ACD in bags and then ACD in bottles. There were no significant differences in ATP concentrations (Fig. 3).

Red blood cell ATP, DPG, and the blood-preservative pH decreased significantly ($P<0.01$) over the 8 weeks of storage. Further analysis disclosed significant dog to dog variation in blood-preservative pH ($P<0.05$), and the DPG concentrations ($P<0.01$). Significant relationships were found between the 3 parameters: comparing blood-preservative pH to ATP ($r=0.55$) and to DPG ($r=0.66$) revealed significant correlations ($P<0.01$). Also, when ATP was compared to DPG ($r=0.75$) a similar result was found ($P<0.01$).

## DISCUSSION

The importance of ATP, DPG, and the pH of stored human blood is their relationship to the viability, function, and clinical usage of the blood. Similar associations have been described for stored canine blood and thus, it is now possible to make preliminary evaluations of storage media and containers based on these *in vitro* biochemical criteria.[5] Final judgements should not be made until *in vivo* testing confirms these results.

The observed significant decreases in ATP, DPG, and pH,

as well as the significant interrelationships of these values, confirm the results of previous experiments.[5] Also, individual differences among the dogs were present.

CPD differs in several respects from ACD solution: 1) CPD contains less citric acid and includes sodium phosphate; 2) the pH of CPD (5.60) is higher than ACD (5.00); 3) it is isotonic to human red cells, whereas ACD is hypotonic; 4) CPD-plasma requires only one third as much base as ACD-plasma to bring the pH within normal values in man.[6] There have been conflicting reports in human literature as to the ability of CPD to maintain the minimum National Institutes of Health standard of 70% red cell viability for 4 weeks storage rather than the 3 week limit which applies to ACD.[2,6,14,16] Since CPD does maintain higher DPG levels, transfused blood functions better in the delivery of oxygen to tissues.[4,12] The elevation of DPG is apparently due to the higher pH of the medium rather than the presence of phosphate ion.[12]

It now appears that CPD is a better storage medium for canine blood. Significantly higher DPG levels should result in improved function of the red cells in the recipient's circulation. The pH differences are a major consideration when contemplating transfusions, especially with large volumes, to patients which may already be acidotic due to clinical conditions. The question of possible increased viability and storage life remains to be resolved, although improvement would be

anticipated based on the high correlation of these *in vitro* parameters to viability.

The lack of significant differences in ATP concentrations among the storage media may not be as critical a consideration as in man. As judged by the respective correlation coefficients of ATP to viability, ATP is not as important in the maintenance of viability of the red cells.[5] Also, canine red cells have only one half as much ATP as human red cells.

We found plastic bags better than the vacuum bottles for the following reasons: 1) excessive foaming and possible extra stress produced by vacuum bottles were not present with the gravity filled bags; 2) the attached collecting tubing and needle with the bags were convenient; 3) the bags were non-breakable; 4) they required smaller storage space before and after collection. One questionable factor with the bags is the migration of the phthlate ester plasticizer from polyvinyl blood bags into stored canine and human blood.[9] This chemical was found in human tissues after transfusions, but its toxicity is not known. Alternative formulations for the production of these bags may be forthcomming.

Objectively, our data indicates the bags would be preferred because of the clinical importance of higher pH in the blood-preservative mixture. Also, increased viability of human red cells has been reported when bags were used instead of bottles.[15]

Therefore, it is recommended that canine blood be collected and stored in CPD solution contained in plastic bags.

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Fig. 1- DPG concentrations of canine blood stored in ACD-bottles, ACD-bags, and CPD-bags: standard deviation of a mean (S.D.) = 1.47.

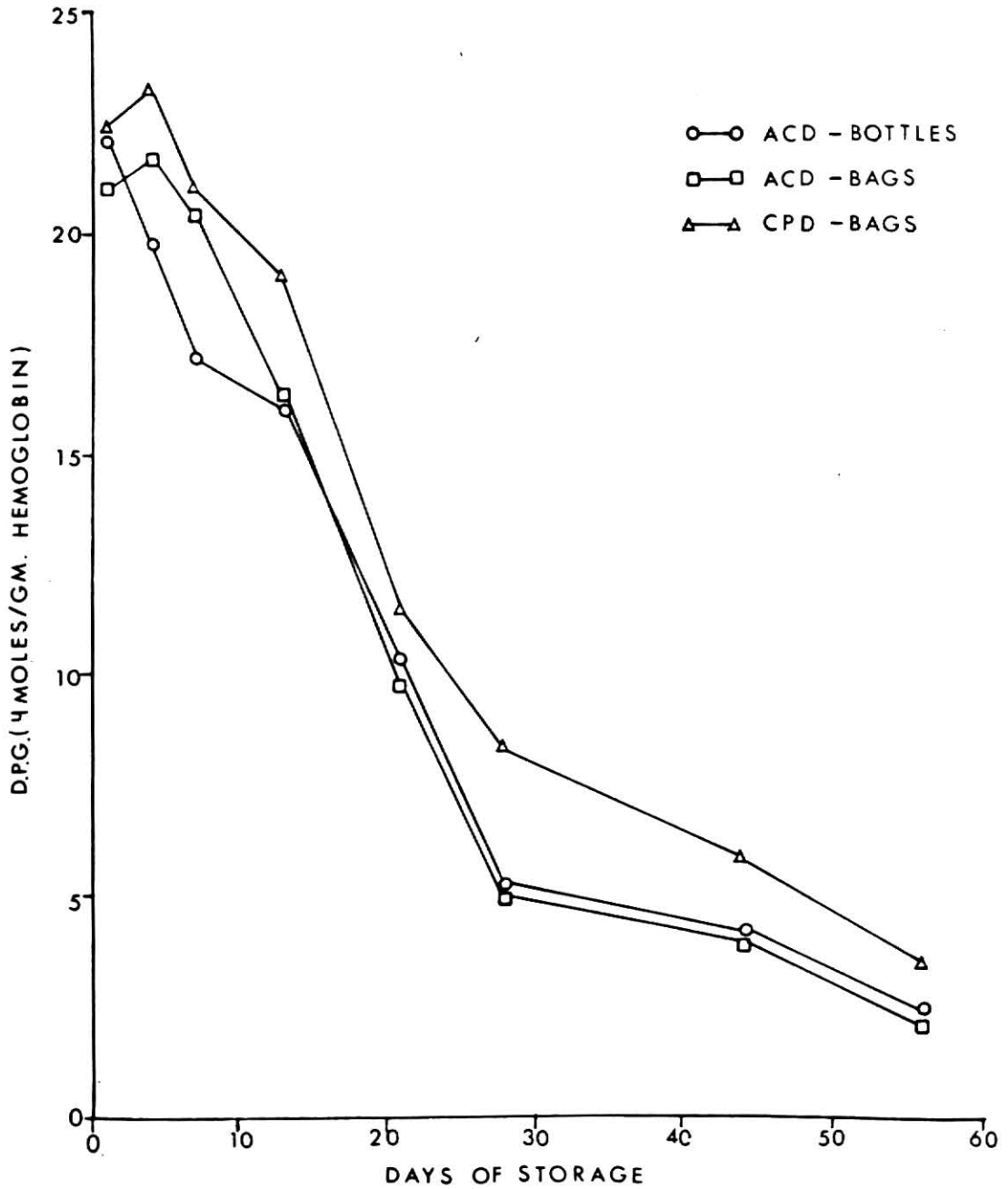

ACD - BOTTLES
ACD - BAGS
CPD - BAGS
D.P.G.( μ MOLES/GM. HEMOGLOBIN )
DAYS OF STORAGE
0
5
10
15
20
25
0
10
20
30
40
50
60

Fig. 2- pH levels of canine blood stored in ACD-bottles, ACD-bags, and CPD-bags (S.D.=0.03).

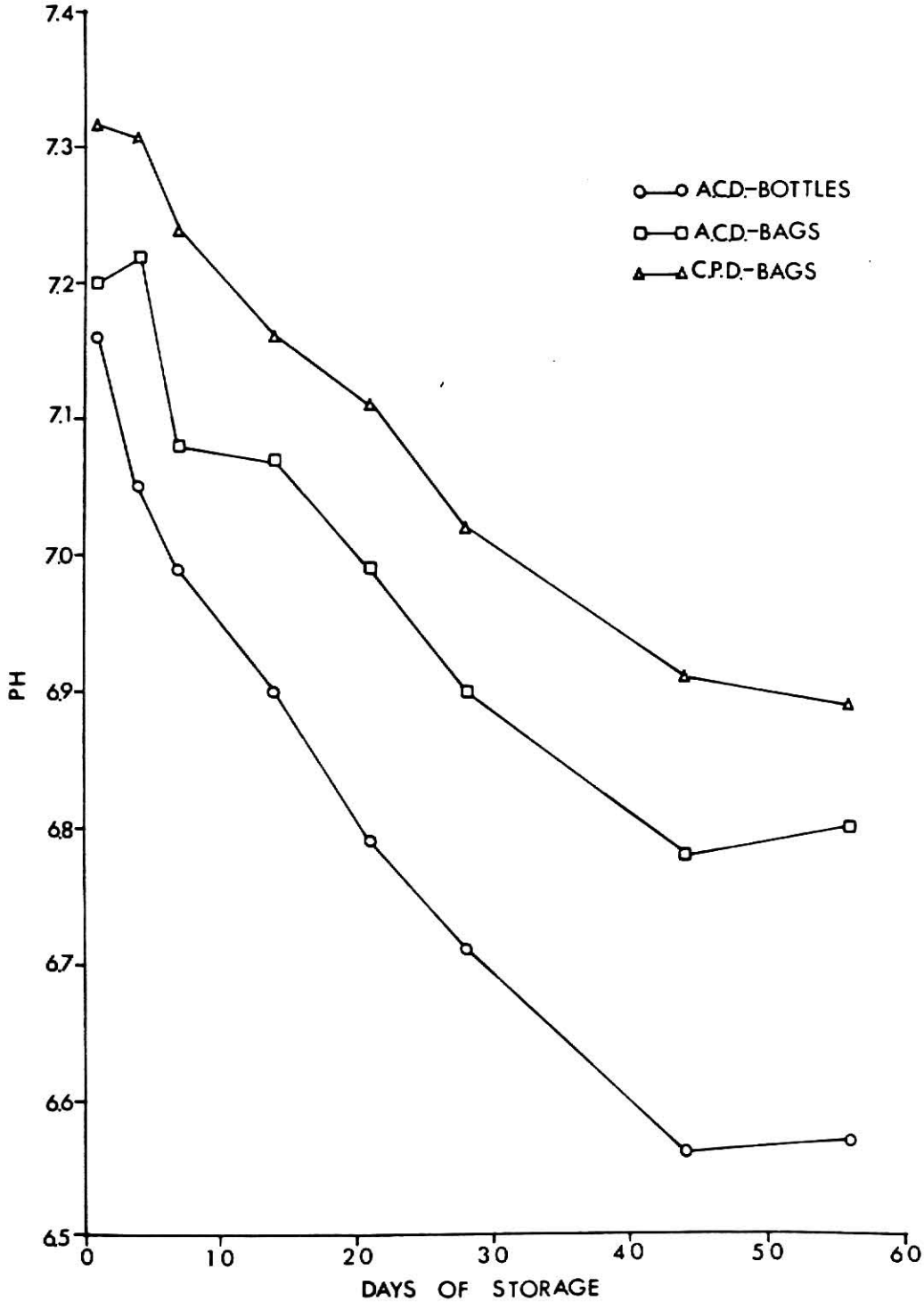
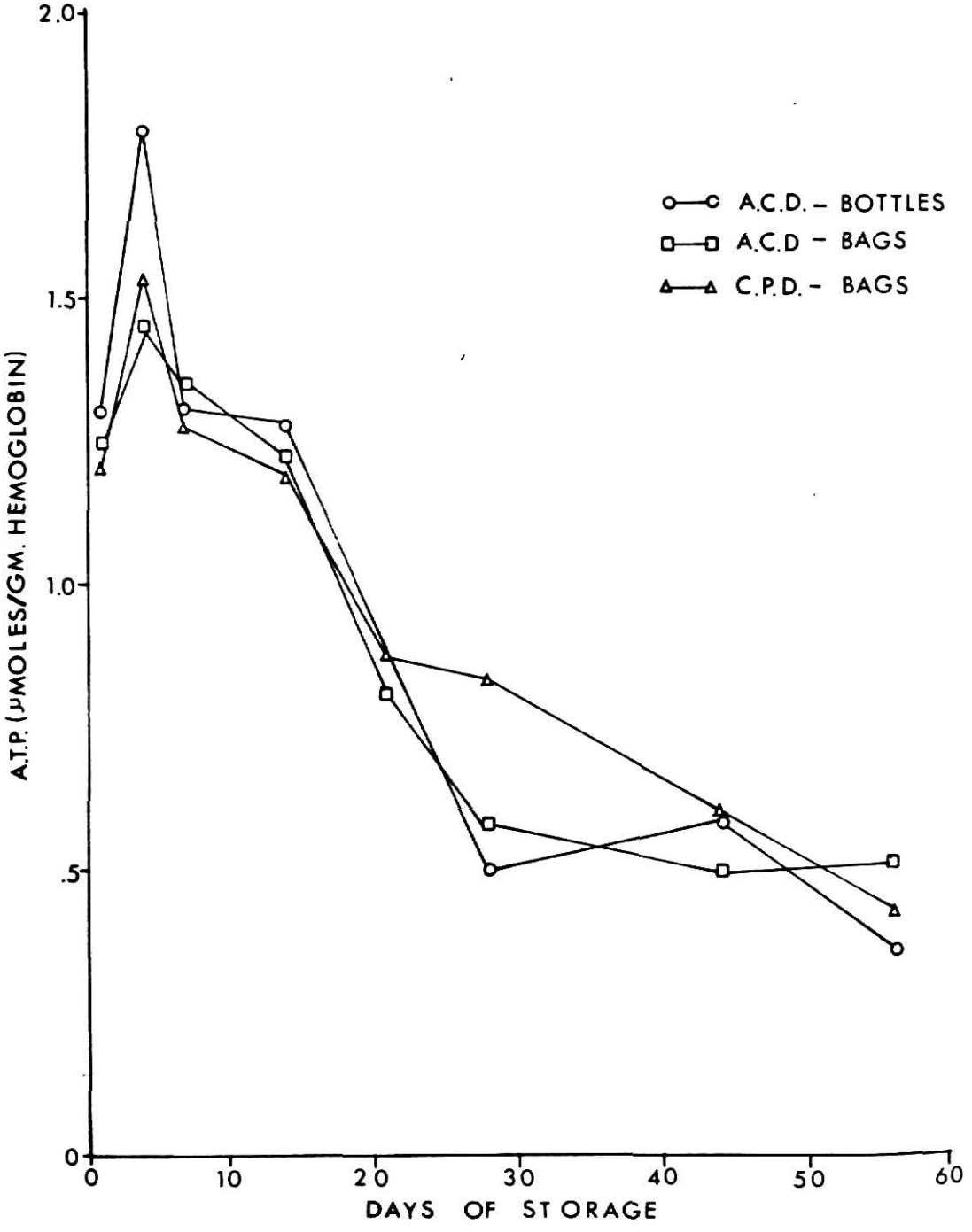

7.4
7.3
7.2
7.1
7.0
6.9
6.8
6.7
6.6
6.5
PH
0
10
20
30
40
50
60
DAYS OF STORAGE
A.C.D.-BOTTLES
A.C.D.-BAGS
C.P.D.-BAGS

Fig. 3- ATP concentrations of canine blood stored in ACD-bottles, ACD-bags, and CPD-bags (S.D.=0.01).


2.0
1.5
1.0
.5
0
A.T.P. (μMOLES/GM. HEMOGLOBIN)
0
10
20
30
40
50
60
DAYS OF STORAGE
A.C.D. – BOTTLES
A.C.D – BAGS
C.P.D. – BAGS

# PAPER 3: EFFECTS OF VARIOUS ADDITIVES ON *IN VITRO* PARAMETERS RELATED TO VIABILITY AND FUNCTION OF STORED CANINE BLOOD

## SUMMARY

The efficacy of various chemical additives combined with acid-citrate-dextrose (ACD) and citrate-phosphate-dextrose (CPD) solutions for preserving canine whole blood was determined. The criteria used to judge the additives were the red blood cell content of adenosine triphosphate (APT) and 2,3-diphosphoglycerate (DPG). An adenine-phosphate-pH 6.5 mixture added to the preservative solution best maintained these chemicals which are associated with viability and functional capacity of the red blood cells. Pyruvate-pH 6.5 and ascorbic acid additives also were beneficial. CPD solution was more effective than ACD for the preservation of canine blood.


## INTRODUCTION

The ability of a preservative to maintain red cell viability and function is the basis on whcih efficacy in blood storage is determined. These important criteria can be estimated in dog and man by <u>in vitro</u> determinations of red cell concentrations of ATP and DPG.[2,8,11,14,31] These organic phosphates decrease during storage of whole blood in both species; consequently the red cell viability and functional capacity to release oxygen to the tissues are also diminished.[14,21,25,34] The first generally used blood preservative, ACD, was developed by Loutit et al.[17] Recently extensive research with human blood has developed media which better

maintained ATP and DPG during storage. Two such solutions, ACD-adenine and CPD, are now used clinically.[1,16,28] CPD solution has been reported to be preferable to ACD for the preservation of canine blood.[15]

The present study was undertaken to determine the relative efficacy of several chemical additives in maintaining levels of ATP and DPG in stored canine blood.

## MATERIALS AND METHODS

Seven solutions were tested as paired samples in ACD and CPD media[a] (Table 1). Concentrated additive solutions were prepared in demineralized water, the pH adjusted when indicated, and then sterilized by passage through a 0.2 micron membrane filter.[b] Measured quantities were then added to the 2 media in amounts which would give the appropriate blood-preservative concentrations. The final test solutions were aseptically injected into 10 ml. vacuum test tubes.[c] Volumes of 1.9 ml. of ACD mixture and 1.17 ml. of the CPD mixture were used to collect a total volume of 9.5 ml. of blood. ACD and CPD media without additives were used as controls in each study.

The experiment was divided into 3 trials. Blood from 4

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[a] Abbo-vac[R] and Pliapak[R]450, supplied by Abbott Laboratories, North Chicago, Ill.

[b] Metricel[R], Gelman Instrument Co., Ann Arbor, Michigan

[c] Vacutainer[R], (#4700), Becton-Dickinson, Rutherford, N. J.

mixed-breed dogs, weighing 15-20 kg., was collected to provide 4 replications in the first study. The second and third studies were designed with 2 replications using blood from each of 2 dogs. The animals were anesthetized and the blood collected by jugular venipuncture. The test tubes were filled to a marked volume, promptly chilled, then stored at 4° C. for 6 weeks.

Approximately 2 hours after collection, and thereafter once a week, the test tubes were gently rotated and a 1 ml. aliquot aseptically withdrawn. Red cell ATP and DPG were measured by standard[3] methods using chemical reagents obtained from Sigma Chemical Company, St. Louis, Missouri. Blood-preservative pH was determined anaerobically at 4° C. using a micro-blood sampling unit.[d] The pH meter was calibrated at the same temperature.

The data from each of the 3 experiments were tested by analysis of variance with the various preservative solutions as the main factors. The effects of weeks, media (ACD and CPD), and individual dogs were also determined.

## RESULTS

Several of the tested solutions maintained higher DPG concentrations than the controls (Fig. 1). Ascorbic acid significantly elevated ($P < 0.05$) the concentration of this organic phosphate at 3-6 weeks of storage. Adenine-phosphate-

[d] Micro Blood Assembly, Beckman Instruments, Inc., Fullerton, California

pH 6.5 and pyruvate-pH 6.5 also maintained significantly higher ($P < 0.01$) levels of DPG. Other additives produced results which were not statistically different from the controls. Regardless of the additive used, CPD maintained significantly higher ($P < 0.01$) DPG quantities than did ACD media.

ATP (Fig. 2) was better preserved in adenine-phosphate pH 6.5 ($P < 0.01$) than in the controls. Conversely, ascorbic acid was deleterious ($P < 0.01$) to this compound. The other treatments produced no meaningful differences. The effect of the 2 media was questionable; studies 2 and 3 revealed significantly higher ($P < 0.05$ and $P < 0.01$) ATP levels with CPD over ACD, but the results of study 1 were not concurrent.

The pH levels of the blood-preservative mixtures (Fig. 3) were significantly lowered ($P < 0.01$) by the addition of phosphate. The experimental elevation of the preservative pH in the third study significantly raised ($P < 0.01$) blood-preservative pH levels. CPD produced higher pH in the stored blood in study 1 and at weeks 0, 1, 4, and 5 of study 2. Study 3 altered the pH as the treatment and no differences in pH between ACD and CPD were detected.

Red cell ATP and DPG and the blood-preservative pH significantly ($P < 0.01$) decreased in all studies during the 6 weeks of storage (Fig. 1-3). None of the test solutions could completely halt the decline of these parameters.

# DISCUSSION

Canine blood stored with adenine-phosphate at pH 6.5 appears to be the most effective combination in improving the biochemical parameters associated with increased viability and function. The adenine-phosphate-pH 6.5 solution improved ATP levels even in the presence of the usually deleterious high pH. Unlike the results using human cells, these chemicals alone were not capable of eliciting this response. The increase in DPG is assumed to be related to the elevated pH, however pH 6.5 alone did not significantly increase DPG concentrations.

Adenine has been used extensively in the search for improved human blood preservatives.[30] This chemical increases ATP levels and viability, and thus, extends shelf life. Adenine is necessary as a source of adenine moiety for the resynthesis of ATP.[19,20,33] No harmful effects to patients were produced when blood preserved with adenine added was transfused.[29]

The beneficial effects of phosphate on ATP and DPG have been reported.[6,13] Phosphate has also been combined with adenine solutions to improve ATP levels of stored blood.[10,36] Phosphate apparently improves glucose utilization through the hexokinase and phosphofructokinase reactions; also it may stablize pH.[18,22,26]

The ATP and DPG concentrations in stored human blood can be altered by variation in preservative pH.[12,32] The

beneficial effect of CPD for DPG levels is due to the higher preservative pH.[27] ATP concentrations are better maintained at pH 5.0 in ACD and pH 5.5 in ACD-adenine.[4,5] Although increased glycolysis is associated with increased pH, this effect may be less pronounced in canine red cells.[9]

Pyruvate-pH 6.5 maintained DPG in higher amounts than the controls. Although it seems reasonable that this was due to the presence of both factors, each alone did not significantly alter DPG levels. In contrast to human blood, ATP levels were unchanged in this solution. This may be due to differences in ATP generation and function between the species.[15]

Pyruvate has been included in several media designed to regenerate ATP and DPG.[7,13,23] Pyruvate circumvents a block in the glyceraldehyde phosphate dehydrogenase (GAPD) step by the oxidation of reduced nicotinamidedinucleotide (NADH) to nicotineamidedinucleotide (NAD) in the lactic dehydrogenase reaction; NAD is then available for the GAPD reaction.[24] The improved glycolysis results in higher ATP and DPG concentrations.

Ascorbic acid was effective in elevating DPG. It was surprising that this chemical also decreased ATP in red cells, unrelated to pH. This drug has been demonstrated to elevate DPG in stored human blood.[35]

CPD media produced higher pH and DPG levels than ACD. These benefits were expected on the basis of previous experiments.[15] CPD also maintained higher ATP levels in the presence of the solutions used in studies 2 and 3. CPD therefore appears to be the standard preservative of choice for the

storage of canine whole blood.

Although the in vitro improvements in stored blood produced by adenine-phosphate at pH 6.5, pyruvate at pH 6.5 and ascorbic acid are preliminary results, they should correlate good with succeeding animal trials. The extension of permissible storage time resulting from better maintenance of viability and increased functional capacity are of obvious benefits to the veterinary profession.

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Table 1- Preservative solutions tested for ability to maintain ATP, DPG, and pH of stored canine blood.

**Fig. 1-** DPG concentrations of canine blood preserved in several additive-media combinations; standard deviation of a mean (S.D.) for study 1 was 1.28, for study S.D.=2.58, and for study 3 S.D.=2.31.

| Study Group | Number of Dogs | Media | | Additives (Micro Moles in Final Blood-Preservative Mixture) | | | | Media pH (at 25° C) |
|---|---|---|---|---|---|---|---|---|
| | | ACD | CPD | Adenine | Ascorbic Acid | Pyruvate | Monobasic Sodium Phosphate | |
| 1 | 4 | + | | | | | | 5.00 |
| | 4 | | + | | | | | 5.60 |
| | 4 | + | | 0.5 | | | | 4.89 |
| | 4 | | + | 0.5 | | | | 5.63 |
| | 4 | + | | | 5.67 | | | 4.92 |
| | 4 | | + | | 5.67 | | | 5.53 |
| 2 | 2 | + | | | | | | 5.00 |
| | 2 | | + | | | | | 5.60 |
| | 2 | + | | | | 3.0 | | 4.85 |
| | 2 | | + | | | 3.0 | | 5.52 |
| | 2 | + | | | | | 30.0 | 4.91 |
| | 2 | | + | | | | 32.0[e] | 5.39 |
| 3 | 2 | + | | | | | | 5.00 |
| | 2 | | + | | | | | 5.65 |
| | 2 | + | | 0.5 | | | 30.0 | 6.50[f] |
| | 2 | | + | 0.5 | | | 32.0[e] | 6.50[f] |
| | 2 | + | | | | 15.0 | | 6.50[f] |
| | 2 | | + | | | 15.0 | | 6.50[f] |
| | 2 | + | | | | | | 6.50[f] |
| | 2 | | + | | | | | 6.50[f] |

[e] CPD media contains 11 mM phosphate

[f] pH adjusted with 10% NaOH

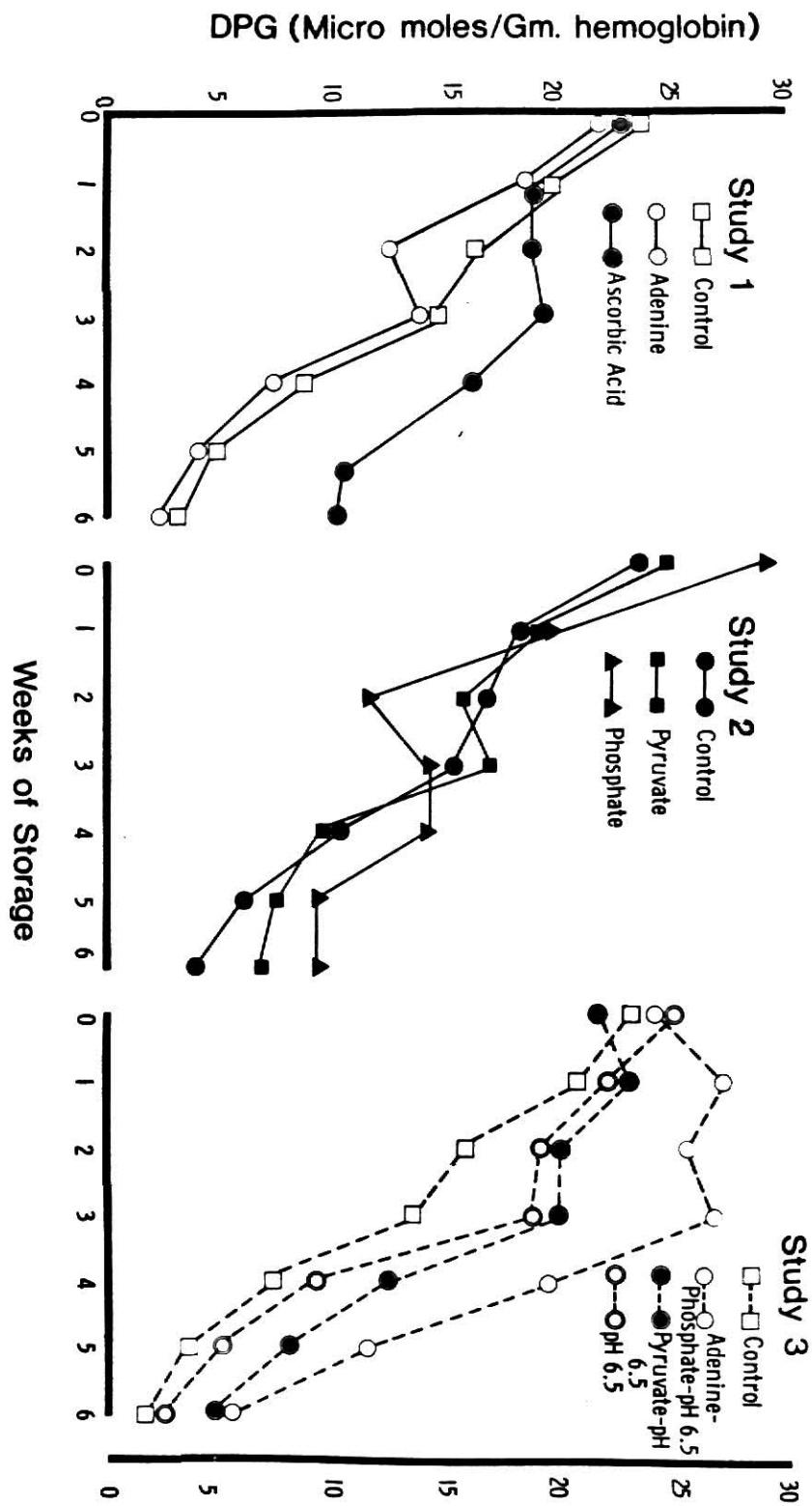

DPG (Micro moles/Gm. hemoglobin)
Study 1
Control
Adenine
Ascorbic Acid
Study 2
Control
Pyruvate
Phosphate
Study 3
Control
Adenine-Phosphate-pH 6.5
Pyruvate-pH 6.5
pH 6.5
Weeks of Storage

Fig. 2- ATP concentrations of canine blood preserved in several additive-media combinations; study 1 S.D.=0.12, study 2 S.D.=0.20, study 3 S.D.=0.16.

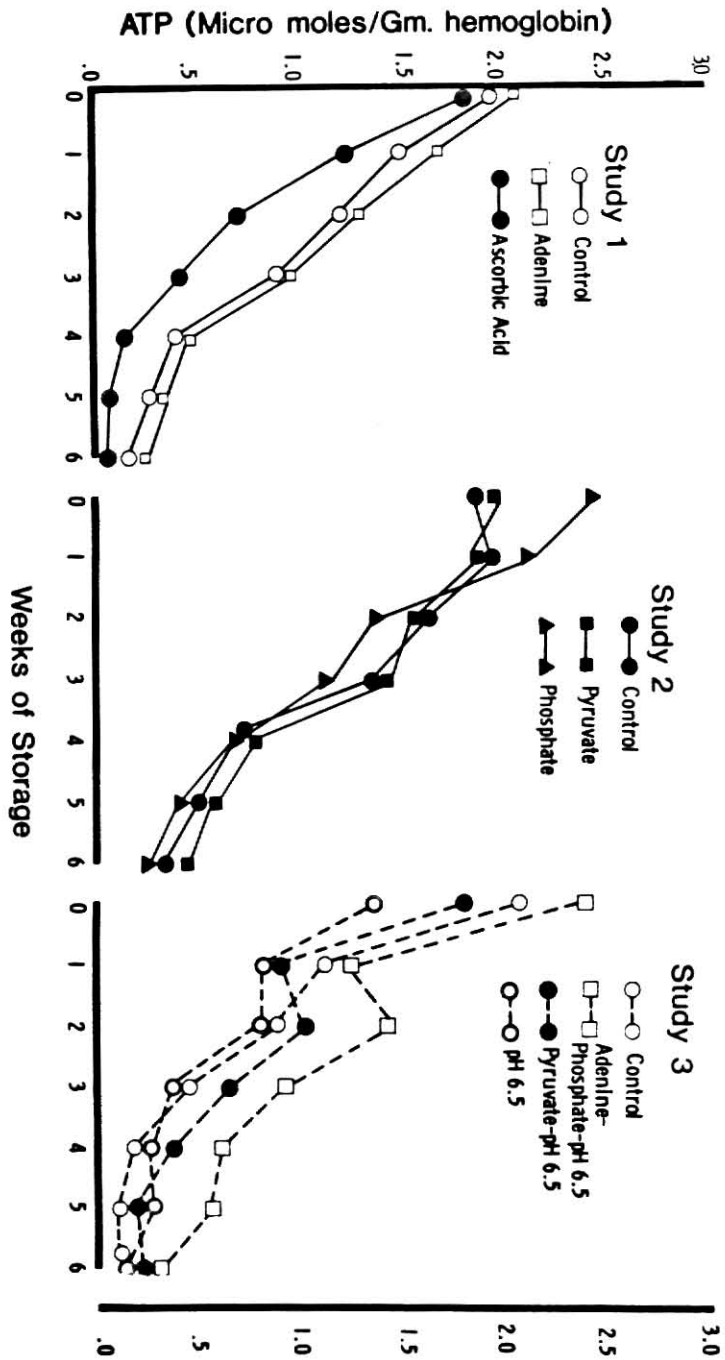
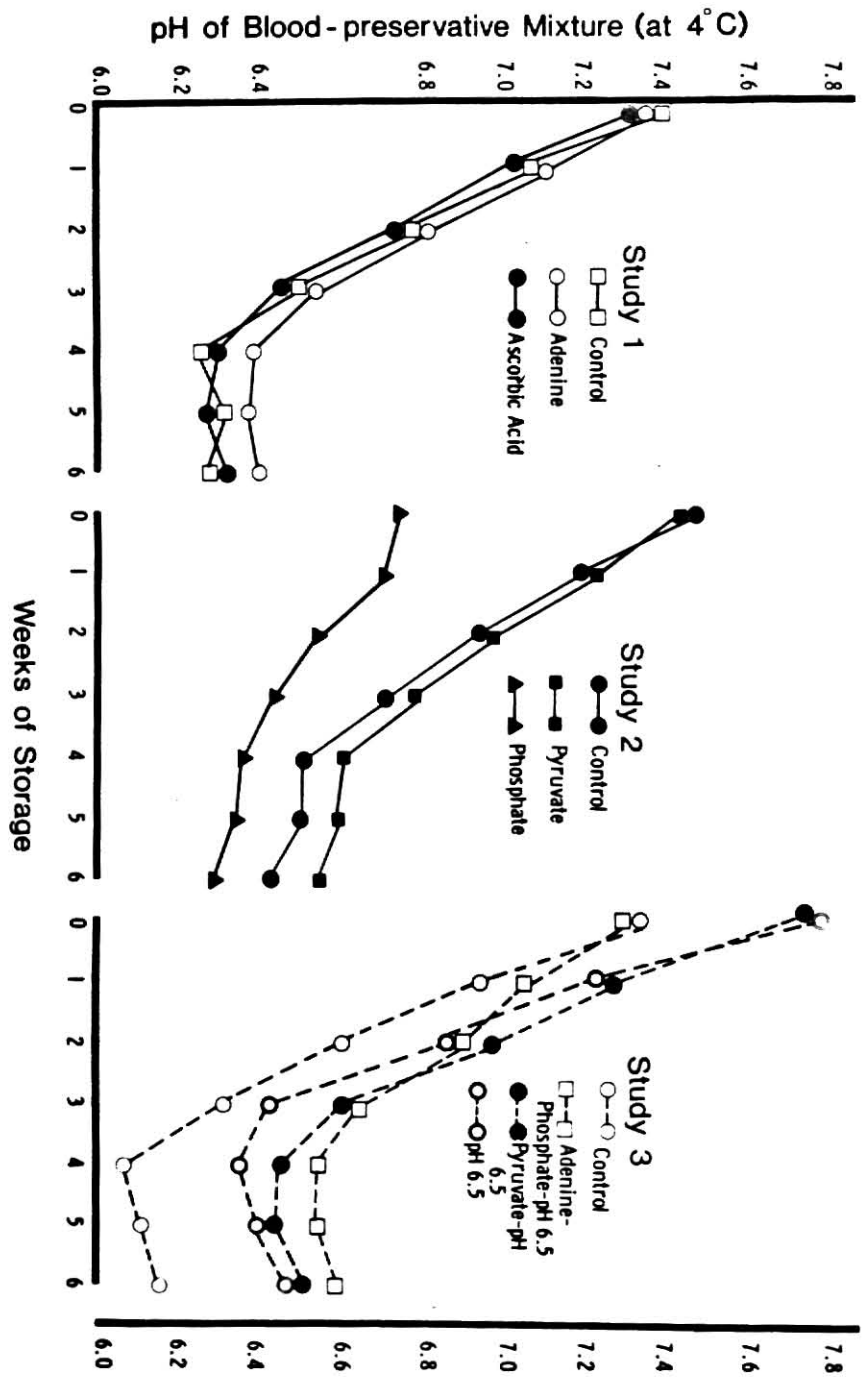

ATP (Micro moles/Gm. hemoglobin)
Study 1
Control
Adenine
Ascorbic Acid
Study 2
Control
Pyruvate
Phosphate
Study 3
Control
Adenine-Phosphate-pH 6.5
Pyruvate-pH 6.5
pH 6.5
Weeks of Storage

Fig. 3- pH levels of blood-preservative mixtures with the inclusion of several additives in the preservative mixtures; study 1 and 2 S.D.=0.04, study 3 S.D.=0.03.


pH of Blood-preservative Mixture (at 4°C)
Study 1
Control
Adenine
Ascorbic Acid
Study 2
Control
Pyruvate
Phosphate
Study 3
Control
Adenine-Phosphate-pH 6.5
Pyruvate-pH 6.5
pH 6.5
Weeks of Storage

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# THE EFFECT OF STORAGE MEDIA ON CANINE BLOOD FOR TRANSFUSION

by

DAVID LEE EISENBRANDT

B. S., Kansas State University, 1967
D. V. M., Kansas State University, 1969

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AN ABSTRACT OF A MASTER'S THESIS

submitted in partial fulfillment of the

requirements for the degree

MASTER OF SCIENCE

Department of Pathology

KANSAS STATE UNIVERSITY
Manhattan, Kansas

1973

# PRESERVATION OF CANINE WHOLE BLOOD FOR TRANSFUSION

## ABSTRACT

Whole blood transfusion has been utilized frequently as a therapeutic procedure in the canine; yet very little is known about the effect of preservative media, the containers, the erythrocyte's functional capacity, or post-transfusion erythrocyte viability. In stored human blood the erythrocyte adenosine-5'-triphosphate (ATP) concentration correlates with post-transfusion viability and 2, 3-diphosphoglycerate (DPG) level determines the affinity of hemoglobin for oxygen. In vitro tests which can be used to predict in vivo performance after storage of canine blood are not available. Once stored blood's performance can be predicted without costly and time-consuming in vivo testing, the effects of various storage media, containers, and chemical additives could be quickly evaluated. The present study was designed to measure erythrocyte ATP and DPG levels, blood-preservative pH, and post-storage viability in canine blood during storage.

The investigations were conducted in 3 parts. In the first experiment, blood was collected into acid-citrate-dextrose (ACD) solution in plastic bags and stored for 6 weeks at 4° C. At weekly intervals, the blood was sampled, and red cell ATP and DPG concentrations, blood preservative pH, and 24-hour post-transfusion viability were determined. In the second experiment canine blood was collected into ACD solution in glass bottles, ACD solution in plastic bags and citrate-

phosphate-dextrose (CPD) solution in plastic bags. Erythrocyte ATP and DPG levels and blood-preservative pH were determined during storage at $4^{\circ}$ C. for 8 weeks. In the third experiment the effect of various additives to ACD and CPD solutions was investigated during 6 weeks of storage.

When canine blood was stored in ACD and CPD media, erythrocyte DPG and ATP levels, post-transfusion viability, and blood-preservative pH decreased significantly. During 6 weeks of storage, erythrocyte DPG and ATP concentrations and blood-preservative pH were correlated with post-transfusion viability. Erythrocyte DPG concentration was the best predictor of cell viability, and did not decrease as rapidly in canine erythrocytes as reported for human erythrocytes. CPD media maintained erythrocyte DPG concentrations and blood-preservative pH at higher levels than ACD solution. The addition of ascorbic acid, adenine-phosphate at pH 6.5, and pyruvate at pH 6.5 to the preservative media maintained higher erythrocyte DPG concentrations than control media. Erythrocyte ATP levels were best preserved by addition of adenine-phosphate at pH 6.5.

Based on this study, the following conclusions can be made. ATP and DPG concentrations of stored canine red blood cells are good *in vitro* tests of *in vivo* viability and functional capacity. Canine blood in ACD solution should not be transfused after 3 weeks of storage. CPD solution is more effective than ACD for the preservation of canine blood.

Canine blood should be collected and stored in CPD solution in plastic bags. The combination of adenine-phosphate and the elevation of the pH to 6.5 in CPD solution is superior to currently available storage media.