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**Hemoglobin Function in Stored Blood**

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**ABSTRACT**
These manuscripts present ATP, 2,3-DPG, pH, and glucose levels of whole blood and packed cells studied in CPD-adenine with the following variables: pH, glucose concentrations, adenine, inosine, methylene blue, pyruvate, dihydroxyacetone, ascorbic acid, fructose, mannose, and ribose.
INTRODUCTION

This report covers 17 months. During the first five months covered which represented an extension of the contract covered in the last submitted, fiscal 1976, annual report and the renewal contract for the 12 months of calendar year 1977: seven sets of experiments have been brought to completion, six sets of experiments were begun and completed, and seven other projects or sets of experiments have been begun and are not completed. The 13 completed sets of experiments are given here and, as well, results of the experiments completed from the seven on-going projects.

All of these experiments are aimed directly at maintaining red cell 2,3-DPG levels during blood storage in order for transfused blood to deliver oxygen to the tissues immediately upon transfusion, regardless of the duration of storage. The background references to the subject of oxygen transport and papers covering previous work on the metabolic regulators and nutrients as well as the basic preservatives used in this study will be found as references in the manuscripts included as appendices to this report. These studies include a definitive, statistical study determining the optimal inosine concentration for 2,3-DPG maintenance over 35 days of storage in a CPD-adenine preservative. Also, a complex study using statistically significant numbers of whole blood units stored as whole blood, soft packed cells, and hard packed cells with 4 different concentrations of extra glucose were performed. Fructose, mannose, ribose, and xylose were studied as alternate sugars to glucose and ribose and xylose as additional sugars with and without inosine and oxygen. The results of eight experiments utilizing ascorbate as the natural vitamin and an optical isomer and sulfhydryl inhibitors are recorded. Ascorbate is the most promising new natural biologic compound for potential use in blood preservation to maintain 2,3-DPG levels. However, its mechanisms
of action have not been clarified and these experiments present my first major attempt to elucidate why it maintains 2,3-DPG levels so well.
RESULTS AND DISCUSSION

Maintenance of normal 2,3-DPG values for 28 days was attained in a CPD-adenine preservative with 10 mM inosine. The 10 mM mean value was significantly higher than the 5 mM value. Higher concentrations of inosine, 15 and 20 mM, were not significantly better at 28 or 35 days compared to 10 mM (Appendix 1).

In hard packed, 80-90% hematocrit red cells, ATP maintenance was adequate with 175 and 200% glucose; whereas, lower concentrations down to 100% of glucose found in regular CPD gave definitely lower ATP values (Appendix 2). Late in blood storage with packed cells, glucose levels have been shown to run low or become depleted when the starting glucose is 100% or 125% of that present in regular CPD. In this experiment 200% glucose provided the only significantly higher ATP levels compared to the other preservatives, this was at 42 days of blood storage. It is also of note that in this experiment glucose concentrations in the hard packed units remained highest during the last three weeks of storage with 200% glucose and the value was statistically significantly higher than most of the lower concentrations. Further, the pH values in the 200% glucose preservatives were the lowest of all of the preservatives from 7-42 days. And, at 28, 35 and 42 days a lower pH value in 200% glucose was statistically significantly lower than the values in 100% glucose. It is clear that more glucose is being utilized in the highest glucose preservative; this is reflected in the lower pH values and in better maintenance of ATP.

When "alternate sugars" to glucose, ribose and xylose were used in citrate-phosphate preservatives, these 5 carbon sugars were seen to give low DRG and ATP values and the pH failed to continue to decrease after 21 days indicating failure of metabolism (Appendix 3). However, with alternate 6 carbon sugars, fructose maintained ATP better than dextrose or glucose, and mannose maintained
better ATP levels but unfortunately worse 2,3-DPG levels and less ATP when inosine was present as well. However, xylose (20 mM) added to a CPD-adenine preservative maintained better 2,3-DPG levels than CPD-adenine alone.

The unnatural D isomer of ascorbic acid was shown to be as effective as the natural L isomer or Vitamin C in maintaining 2,3-DPG levels. However, both forms had an adverse effect on ATP maintenance. These results suggest a mechanism of metabolic regulation for ascorbate as the reason for its effects on ATP and 2,3-DPG levels. Only the natural L isomer or vitamin form would be expected to be effective and incorporated if ascorbic acid were being metabolized as a metabolic nutrient. Ascorbic acid is a complex 6 carbon sugar, a gluconolactone.

As a redox compound, ascorbic acid is posited in red cell metabolism as a self regenerating oxidant of NADPH to NADP. The result of this might be acceleration of glucose oxidation via the pentose phosphate pathway giving increases in DPG levels during blood storage. Utilization of the pentose phosphate pathway during blood storage in order to get carbon sugars into 2,3-DPG is appealing because the most rate-lmiting enzymic step in glycolysis under the conditions of liquid blood storage, phosphofructokinase, is bypassed. This mechanism has been used before by this investigator to explain the mechanism of action of the most useful model compounds which maintain 2,3-DPG, the metabolic nutrient, inosine and the metabolic regulator, methylene blue. Further, ascorbic acid's effect if it is that of oxidizing NADPH would be analogous to methylene blue's effect which has been clearly shown to be mainly that of oxidizing NADPH. In considering ascorbate's likely role in the pentose phosphate pathway one must remember that the red cell maintains its ability to keep sulfhydryl groups in their reduced functional states via the pentose phosphate pathway, as it lacks an oxidative citric acid cycle. This limit to the red cell's metabolic protective mechanisms, allows study of the mechanisms of ascorbate's maintenance of 2,3-DPG using gluta-
thione and sulfydryl inhibitor reagents. Some of these studies have shown that added glutathione depresses DPG maintenance but with ascorbate the depression is removed (Appendix 4). So, since 2,3-DPG is better maintained with ascorbate in the presence of the sulfydryl blocking reagents NEM and iodoacetate, one can suggest that with the competition for utilization of ascorbate by sulfydryl groups having been removed ascorbate is allowed to better stimulate the pentose phosphate pathway thus maintaining 2,3-DPG, compared to when the inhibitors are present. GSH and inhibitor experiments were done with the unnatural D isomer. Experiments with both forms show better ATP maintenance with L ascorbate, the natural vitamin, indicating a slight but significant nutritional role for ascorbate in red cell metabolism.
SUMMARY

The multi-institutional CPD-adenine clinical trial for 35 days of storage of whole blood and packed cells using 125% glucose has been completed and in Oct. 1976 at the combined NIH-FDA meeting the Advisory Panel on Efficacy of the Bureau of Biologics, Div. of Blood and Blood Resources unanimously approved this new blood preservative for 35 days of storage. Studies on platelet survivals and a new plastic to be used with this preservative are being completed early in 1978. This preservative will be licensed. There is great interest in a slightly better preservative that will have higher glucose concentrations and perhaps slightly higher adenine concentrations as well. My large extra glucose study reported here and other studies ongoing will contribute to these developments. My work on the overall adenine project is being coordinated with Blood Research Lab at LAIR and the other Army contractors in the field.

The major effort or mission; that is, maintaining normal 2,3-DPG levels throughout storage has achieved considerable progress noted in this report in defining the best available non-toxic compound or combinations of compounds for this purpose. In the meantime physiologic animal and human studies have showed improved function and survival when higher or normal 2,3-DPG blood is transfused compared to low 2,3-DPG blood. Over 20 published studies which show that a transfusion effect is related to 2,3-DPG levels have been summarized by this investigator in a bibliography. Some of the most significant and clearest studies in animals showed increased survivals when transfused with normal 2,3-DPG red cells, compared to low 2,3-DPG red cells under the stress conditions of hypotension, hemorrhage (hematocrit maintained in 2/3 normal), hypoxia, and regional ischemia. It is significant that the 1977 report of the Director, NIHBI to the President of the U.S. said "for optimal storage of red blood cells, their level of 2,3-DPG maintenance must be maintained".
Appendix 1


Appendix 2


Appendix 3


Appendix 4

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