METHOD AND ADDITIVES FOR IMPROVING THE QUALITY AND SHELF LIFE OF STORED BLOOD

A method for improving the quality and/or increasing the shelf-life of whole blood and red blood cell concentrates during storage thereof comprising manipulating the activities of the key red cell enzymes involved in the biosynthesis and degradation of 2,3-DPG (DPG, DPGP, PGP) and in regulation of glycolysis (PK and PFK). The enzymes are manipulated, i.e. activated or inhibited, either singly or in combination. Such manipulations are achieved by adding to the whole blood or red blood cell concentrates an effective amount of one or more compounds which primarily inhibit the activity of DPG phosphatase and maintain those of phosphofructokinase, phosphoglycolate phosphatase and DPG mutase. Among the compounds which can be used are L-amino acids, free fatty acids, glycolytic intermediates including analogs and derivatives of phosphoenolpyruvate, and free bases, inhibitors of DPGP as well as structural analogs and derivatives of all of the above compounds.
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METHOD AND ADDITIVES FOR IMPROVING THE QUALITY
AND SHELF LIFE OF STORED BLOOD

FIELD OF THE INVENTION
5 The present invention relates to a method for increasing quality and/or shelf life of whole blood and red blood cell concentrates, hereafter both referred to as red cells, stored at refrigeration temperatures during usual blood banking procedures.

10 BACKGROUND OF THE INVENTION
The biochemical processes that occur during blood preservation or storage contribute to the diminution of the post-transfusion viability, i.e. survival of stored red cells after transfusion to a recipient, and oxygen off-loading capacity of red cells, both of which are statistically correlated with the duration of storage period. Previous studies of stored red blood cells have indicated that intra-cellular levels of ATP (adenosine triphosphate) and 2,3-DPG (2,3-diphosphoglycerate) largely or entirely determine the post-transfusion viability and oxygen off-loading capabilities respectively of the red cell. It is well-established that both of these substances are products of glycolysis, a biochemical pathway that degrades glucose.

25 Maintenance of post-transfusion viability of stored red cells is closely correlated with the levels of cellular ATP, a high energy compound. Glycolysis is the only ATP generated pathway in the red cell. It appears that ATP preserves membrane integrity by maintaining proper ionic gradients across the red cell membrane, adequate lipid
turnover rate, hemoglobin in a functional state and normal equilibrium of oxidized and reduced glutathione, along with synthesis of adequate amounts of NAD+, i.e. Nicotanamide adenine dinucleotide and its phosphate.

It is well-established that oxygen off-loading ability of red cells is determined by another glycolytic intermediate, 2,3-DPG. This compound declines as a function of time during storage, most likely secondary to both a decreased rate of synthesis and an increased rate of degradation. The red cells with low 2,3-DPG show increased oxygen affinity or decreased ability to release oxygen at the tissue level. Thus, the stored red cells are less efficient vehicles of oxygen transport, the most important function of the red cells—stated otherwise, this means that stored and therefore 2,3-DPG depleted red cells are of poor quality with regard to their function.

It follows that it would be highly desirable to be able to maintain near-normal red cell 2,3-DPG and ATP as such a result would have profound effects in terms of both red cell function and post-transfusion survivability in vivo. Thus, adequate maintenance of both of these compounds would permit the storage of red cells for a longer period of time, which would alleviate problems of shortage of blood for transfusion and low quality of stored blood.

Previous studies of stored whole blood and stored red cell concentrates have indicated that intracellular levels of ATP and 2,3-DGP are important in extending storage capabilities. To this end, several studies have been conducted which have incorporated various chemical additives along with CPD (citrate-phosphate-dextrose) anticoagulant to
stimulate glycolysis, yielding a net increase in red cell ATP level. One of the commercial additives that has recently been studied is adenine. The incorporation of adenine along with CPD anticoagulant into stored blood appears to increase ADP (adenine diphosphate) levels, thereby driving the glycolytic equilibrium toward the synthesis of ATP. However, adenine has an adverse effect on the maintenance of the level of 2,3-DPG, i.e. it lowers 2,3-DPG level with concomitant poor function of the stored red cells.

Recent concern over the levels of ATP and 2,3-DPG has become a controversial subject. Because the main objective of transfusing patients is to provide or improve the oxygen delivery to the tissues, the blood oxygen affinity, directly determined by 2,3-DPG, is of critical importance. Therefore, in providing patients with suitable blood for transfusion, one must now consider not only red cell viability in vivo but also hemoglobin oxygen affinity for adequate oxygen transport function, the ultimate goal of red cell transfusion. As a result, research has also been geared towards incorporation of chemicals into the CPD and other preservative media to increase 2,3-DPG and ATP levels.

The significance of near-normal 2,3-DPG-containing red cells becomes self-evident when one examines various clinical conditions such as congestive heart failure, right to left cardiac shunts, and hypoxemia due to pulmonary disease, where patients singularly require the oxygen transport function of the transfused red cells. The transfused red cell, totally depleted of 2,3-DGP, is said to regain half the normal level of this substance within about
24 hours, but this increase may not be rapid enough to be effective in severely ill patients. Furthermore, it is not known whether the rate of resynthesis of 2,3-DPG in the donor cells given to a critically ill patient is comparable to that observed in normal recipients. Dennis et al. (Surgery 77 (6):741-747, June 1975) has reported a direct correlation between the ability to compensate for low 2,3-DPG levels and the severity of the illness of the patient. Blood with nearly normal hemoglobin-oxygen affinity is thus preferable for use in massive transfusions, particularly in infants, older patients and patients with complicating cardiovascular and pulmonary disease.

The physiological effects of high oxygen-affinity (2,3-DPG depleted) red cells on the myo-cardial, cerebral, hepatic, and renal functions have not yet been fully evaluated, although patients requiring massive transfusions seem to be most susceptible to the adverse effects due to very low levels of 2,3-DPG; see Beutler et al., Vox Sang. 20:403-413 (1970).

Although numerous investigations indicate that the levels of ATP and 2,3-DGP can be better maintained when the two chief preservative solutions ACD (acid citrate dextrose) and CPD (citrate phosphate dextrose) are supplemented with adenine, inosine, or both during storage at 4°C., this conclusion must be approached with some caution. As has been reported by Bunn et al. in New England Journal of Medicine 282:1414-1421 (1970), a patient receiving three or four units of thus-supplemented blood may develop hyperuricemia, which persists for approximately 24 hours.

As reported by Valeri in J. Med. (Basel) 5(5):278-291
(1974), a further cause for concern is the possible renal toxicity of 2,8-dioxyadenine, a metabolite of adenine. Moreover, additives that maintain ATP level, i.e. adenine, tend to lower 2,3-DPG level and those that maintain 2,3-DPG tend to lower ATP level, thus making the maintenance of both of these compounds a currently unrealizable goal. No matter which chemical is used with an ACD or CPD preservative solution, it appears that only a combination of various chemical additives will maintain 2,4-DPG levels in blood under refrigeration conditions for an acceptable period of time.

Deindoerfer et al, in U. S. patent 3,795,581, disclose a method of storing and preserving whole blood using an aqueous solution of dihydroxyacetone to increase the 2,3-DPG content. In a later patent, 3,874,384, Deindoerfer et al disclose the use of a combination of dihydroxyacetone and ascorbic acid to maintain dpq levels in stored blood. Estep, 4,386,069, discloses the use of a fatty ester having at least two ester linkages comprising fatty hydro-carbon groups of from four to twelve carbon atoms each to enhance the preservation of normal blood cell morphology during storage. Harmening, 4,112,070, discloses a process for extending the useful shelf life of red cells by maintaining adequate levels of ATP and 2,3-DPG by adding an insoluble polymer material as a source of inorganic phosphate ions during the storage period. Harmening-Pettiglion, 4,390,619, discloses a method for extending the shelf life of blood platelets by maintaining both the pH and ATP levels suitable for transfusion. This is accomplished by providing to the platelets a water-insoluble polymer containing releasable
weakly basic buffer ions capable of continuously supply
buffer to the platelets.

BRIEF SUMMARY OF THE INVENTION

It is, accordingly, an object of the invention to
overcome deficiencies in the prior art, such as indicated
above.

Another object is to provide for the improved storage
of red blood cells.

Further objects are to provide a method for increasing
the quality and/or shelf life of red blood cells; and to
provide additives to accomplish same.

These and other objects according to the present
invention are achieved by a method for improving the oxygen
off-loading capacity and post-transfusion viability of whole
blood and red blood cell concentrates and thereby extending
the shelf life of the same stored at refrigeration
temperatures or otherwise during normal blood banking. The
shelf life of the blood is increased by increasing ATP and
2,3-DPG levels through manipulation (activation or
inhibition) of red blood cell glycolytic and non-glycolytic
enzymes during storage. This manipulation consists of
inhibiting the activities of the enzyme pyruvate kinase (PK)
and 2,3-DPG phosphatase (DPGP) and activating or maintaining
the activities of phosphofructokinase (PFK), 2,3-
diphosphoglycerate mutase (DPGM) and phosphoglycolate
phosphatase (PGP).

Inhibition of PK and activation or maintenance of DPGM
constitute the major thrusts of the present invention,
whereas activation or maintenance of PFK and PGP, and
inhibition of DPGP constitute the secondary thrusts. The
enzymes are manipulated either singly or in various combinations to optimize the maintenance of 2,3-DPG and ATP levels. The manipulation of the enzymes is brought about by using a variety of compounds which are permeable to the red blood cell membrane either naturally or under certain artificial conditions. These compounds are either known or potential effectors, i.e. activators and inhibitors of the above-mentioned enzymes. Among all the enzymes mentioned above, it has been found that the enzyme PK is strategically located in the glycolytic pathway with regard to 2,3-DPG and ATP production and therefore appears to provide a sensitive point of control to manipulate red cell glycolysis.

The location of PK in the glycolytic pathway allows for the possibility of establishing a glycolytic shuttle (a reversible glycolytic flux above the PK step) that maintains 2,3-DPG and ATP levels by partially inhibiting the enzymatic activity of PK. A partial inhibition of PK results in phosphoenolpyruvate (PEP) not being further catabolized. As the level of PEP builds up, it is shuttled backwards to generate 1,3-DPG which is then converted to 2,3-DPG. The biosynthesis and degradation of 2,3-DPG are normally primarily controlled by the level of 1,3-DPG (which is a function of the overall glycolytic flux) and by the activity of 2,3-DPG phosphatase (DPGP). However, a buildup of 2,3-DPG due to a partial inhibition of PK puts the level of 2,3-DPG in the position of regulating the glycolytic flux by a negative feedback. Any reduced ATP generation as a result of reduced PK activity may be overcome by driving the ATP salvage pathway through addition of adenine.

Since the inhibition of PK is only partial, ATP necessary to
drive the salvage pathway will be available and an oscillating glycolysis will be established in the red cell. The levels of ATP and 2,3-DPG will oscillate within normal ranges, thus preserving the viability and function of the red blood cells despite prolonged storage.

In order to potentiate the effect of inhibitors of PK, it is also desirable to manipulate other glycolytic and non-glycolytic enzymes mentioned above. The activity of DPGM in particular is directly correlated to both storage time and the level of 2,3-DPG, i.e. DPGM activity and 2,3-DPG levels decline proportionately as a function of storage period. These results indicate that the maintenance of DPGM level helps maintain 2,3-DPG level. Since PFK controls the overall glycolytic flux, and especially permits the flux above the 2,3-DPG shunt and therefore determines the level of 1,3-DPG (a precursor of 2,3-DPG), maintenance of its activity helps to maintain both 2,3-DPG and ATP levels. DPGP appears to be more active during storage due to low pH of the stored red cells and therefore a selective inhibition of the enzyme will also assist in the maintenance of 2,3-DPG levels. Lastly, an activation of PGP helps reduce the level of glycolate-2-phosphate (G-2-P), a potent activator of DPGP, and therefore increases the 2,3-DPG level.

One of the major advantages of maintaining 2,3-DPG level by manipulation of red cell enzymes as described above is that it reduces lactic acid production by the red blood cells. This reduces lactic acidosis of the preservation medium and its inhibitory effects on glycolysis (primarily at the PFK and DPGM steps) and its detrimental effects on a number of other cellular processes. Compounds which are
either known to inhibit pyruvate kinase or would be expected
to do so (both experimentally and biologically) are
numerous, as compared to those known to affect other above-
mentioned enzymes. Some of these compounds have overlapping
additive or conflicting influence on these enzymes. For
these compounds to be useful in preserving blood, they must
also be capable of permeating the red blood cell membrane,
must be able to exert their effect preferably at low
concentrations, and above all, be physiologically
acceptable, so that they can be used in humans in vivo. The
following Tables enumerate some of the compounds found to be
useful in inhibiting PK (Table #1) and activating or
inhibiting PFK, DPGM, DGPF and PGP (Table #2). Compounds
that are inhibitors of PK may be classified into five groups
as follows; including the concentration in the stored blood
that are effective:

**Table #1 (PK Inhibitors)**

<table>
<thead>
<tr>
<th>Group I: L-Amino Acids and their derivatives and Structural analogs.</th>
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<tr>
<td>L-methionine - 1-10mM</td>
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<tr>
<td>Methyl a-amino-isobutyric acid -1 -2-mM</td>
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<tr>
<td>cycloleucine - 1-2mM</td>
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<tr>
<td>methylcysteine - 1-2mM</td>
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<tr>
<td>ethylcysteine - 1-2mM</td>
</tr>
<tr>
<td>L-alanine; L-1-aminoethyl phosphoric acid</td>
</tr>
<tr>
<td>L-phenylalanine; L-phenylalanine methyl ester; L-1-amino-2-phenylethyl phosphoric acid - 0.5-10mM</td>
</tr>
<tr>
<td>L-cysteine - 1-2mM</td>
</tr>
<tr>
<td>a-amino-isobutyric acid - 0.5-5mM</td>
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Group II: Free Fatty Acids, their Derivatives and Structural analogs.
- Octanoate - 0.2-5 mM
- Laurate - 0.2-5 mM
- Linoleate - 0.2-5 mM
- Palmitate - 0.2-5 mM
- Myristate - 0.2-5 mM
- Elaidate - 0.2-5 mM

Group III: Glycolytic Intermediates and their Structural Analogs and Derivatives.
- Phosphoenolpyruvate - 0.5-2 mM
- D-Phospholactate - 20-50 µM
- D-Phosphopyruvate - 0.5-2 mM
- Phenyl pyruvate - 0.5-2 mM
- 2-phosphoglyceric acid - 2-10 mM
- 3-phosphoglyceric acid - 2-10 mM
- Phosphoglycolate - 50-100µM
- Phosphoglyoxylate - 50-100µM
- Glyoxylate - 0.5-2 mM

Group IV: Free Bases and their mono, di and triphosphates, their Derivatives and Structural analogs.
- Adenosine (AMP, ADP, ATP) - 0.5-2.5 mM
- Inosine (IMP, IDP, ITP) - 0.5-2.5 mM
- Cytidine (CMP, CDP, CTP) - 0.5-2.5 mM
- Thymidine (TMP, TDP, TTP) - 0.5-2.5 mM
- Guanosine (GMP, GDP, GTP) - 0.5-2.5 mM
- Uridine (UMP, UDP, UTP) - 0.5-2.5 mM
- Mono- and dibutyryl cAMP - 0.1-0.5 mM
- cAMP - 0.1-0.5 mM
\textsuperscript{N\textsuperscript{6}}-(Phenylisopropyl)

\begin{itemize}
  \item Adenosine \hspace{1cm} - 10 \mu M
\end{itemize}

\textbf{Group V: Miscellaneous Compounds and their Derivatives and Structural analogs.}

\begin{itemize}
  \item Ammonia \hspace{1cm} - 0.1-0.2 \mu M
  \item Glucagon \hspace{1cm} - 0.1-0.2 \mu M
  \item Acetyl co-A and its derivatives \hspace{1cm} - 30-200 \mu M
  \item Allantoin \hspace{1cm} - 1-2 mM
  \item Uric acid \hspace{1cm} - 1-2 mM
  \item 4-ethylxaloacetate \hspace{1cm} - 10-20 \mu M
  \item Phenylethylbiguanide and its analogs \hspace{1cm} - 1-2.5 mM
  \item Quercetin \hspace{1cm} - 10-100 \mu M
  \item Epinephrine (\pm Ro 1724) \hspace{1cm} - 1-20 \mu M
  \item Copper \hspace{1cm} - 0.1-2 \mu M
  \item Phosphate, phyrophosphate \hspace{1cm} - 1-20 \mu M
  \item Pru-1, 6-diphosphate \hspace{1cm} - 1-20 \mu M
  \item Oxylate \hspace{1cm} - 2-mmol
\end{itemize}

\begin{table}[h]
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\caption{Table 2 (Effectors of PFK, DPGM, DPGP and PGP)}
\begin{tabular}{ll}
\hline
Activators of PGP & Cobalt \textsubscript{SO_4} (up to 10 \mu M) \\
& Magnesium Chloride (up to 10 \mu M) \\
& Nickel Sulfate (up to 10 \mu M) \\
\hline
Inhibitors of DPGP & Glycerate-2-P 50-100 \mu M \\
& Glycerate-3-P 50-100 \mu M \\
& Glyoxylate 50-100 \mu M \\
& AMP, cAMP 50-100 \mu M \\
& Pi, PPI 30-100 \mu M \\
& Malonate \hspace{1cm} up to 10 mM \\
& Malate \hspace{1cm} up to 5 mM \\
\hline
\end{tabular}
\end{table}
Maleate up to 10 mM
D-Tartrate up to 10 mM
L-Tartrate up to 10 mM
meso-Tartrate up to 10 mM
Glutamate up to 15 mM
D-L-isocitrate up to 1 mM
D-isocitrate up to 1 mM
trans-aconitate up to 0.5 mM
cis-aconitate up to 0.20 mM
Phyrophosphate up to 0.050 mM

Presently, red blood cells stored in best available preservative media that have a normal survival afforded by adequate ATP levels are unable to deliver as much oxygen as are fresh red blood cells due to decreased 2,3-DPG concentrations (see Figure 1). The present invention allows for the improved maintenance of 2,3-DPG and ATP levels during storage been characterized by the FDA as being safe. These substances also will not significantly alter such blood physical characteristics as pH or flow rate.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows levels of 2,3-DPG, ATP, 2,3-DPG mutase, PFK, 2,3-DPG and DPG mutase, and ATP and PFK in blood over a period of time.

Figure 2 shows reactivation studies of DPG mutase with different activators.

Figure 3 shows levels of glucose, glucose-6-phosphate, pyruvate, fructose-6-phosphate, lactate, and pH in blood over a period of time.

Figure 4 shows the standard curve for the radiometric assay of glycolate-2-P.
DETAILED DESCRIPTION

The biosynthetic and biodegradative mechanisms that control the level of 2,3-DPG during red cell storage, with special reference to the activities of glycolytic kinases and 2,3-DPG mutase as components of the synthetic pathway and 2,3-DPG phosphatase and its activator glycolate-2-phosphate (G-2-P) constituting the biodegradative pathway have been investigated. Glycolate-2-P, has been quantitated in normal red cells, since its presence had not previously been unequivocally demonstrated, and the glycolytic enzymes, intermediates and other parameters during prolonged red cell storage (up to 12 weeks) have been studied.

Demonstration of the Existence of Glycolate-2-P, (G-2-P) in Human Red Cells

In order to demonstrate the presence of G-2-P, which had been reported to exist at micromolar concentrations in human red cells in a single brief report, G-2-P from human red cells has now been purified and concentrated using modification of the technique described by Rose and Salon, Biochem. Biophys. Res. Commun. 87:869-875, 1979. The radiometric assay now developed to quantitate G-2-P is extremely sensitive and can detect as little as 50 pmole of G-2-P (Figure 4).

The results conclusively demonstrate that G-2-P does exist in human red cells and therefore may have a regulatory role in the maintenance of 2,3-DPG level during steady state and during red cell storage. These data, therefore, indicate that structural analogs and derivatives of G-2-P are useful as inhibitors of DPG phosphatase.
**Measurements of the Key Glycolytic Enzymes and Intermediates During Storage**

The following red cell parameters during blood storage in ACD, CPD-A1, and Adsol preservatives have now been determined: ATP, 2,3-DPG mutase, PFK, PK, HK, G-6-P, F-6-P, FDP, glucose, lactate, pyruvate, pH and free hemoglobin (Figures 1 and 3). The results are suggestive of the fact that 2,3-DPG mutase appears to become rate-limiting in red cell glycolysis which causes lowering of 2,3-DPG levels as a function of time (Figure 1). The time-course of decline of 2,3-DPG mutase is essentially identical with and superimposable on that of 2,3-DPG. There has now also been observed a time-dependent activation of 2,3-DPG mutase as well as activation of the enzyme due to various activators, especially inorganic Pi (Figure 2). Based on these results, the activity of 2,3-DPG mutase is maintained to better maintain 2,3-DPG levels.

**Inhibition of Glycolytic Flux at the PK Step**

It has been found that the best way to maintain higher 2,3-DPG levels was to metabolically inhibit the pyruvate kinase (PK) reaction. The method of the present invention was tested by similar studies of blood stored with L-alanine and L-phenylalanine, two potent inhibitors of PK, using a series of concentrations. The results indicate that both of these amino acids are effective in better preserving 2,3-DPG levels during blood storage. The time taken for 2,3-DPG to decline to 50% of the original value ($t_{1/2}$) was prolonged from 10 days (no additive) to 13-14 days (with additive).

In addition, the extent of the transport of these amino
acids within the red cell has been quantified under the standard procedures of blood collection, processing and preservation used currently.

Furthermore, the scientific validity of the concept has been confirmed by concurrent investigations of the blood from an individual heterozygous for PK deficiency. This individual possesses one-half normal PK activity in her red cells, and thus provides a natural model to study the effect of inhibiting the enzyme from normal blood. As expected, her red cells showed the best maintenance of 2,3-DPG levels, i.e., a t1/2 of 16-17 days. Thus, it appears that if an additive can bring about ~70-80% inhibition of red cell PK, it will be able to improve the quality and/or increase the shelf life of banked blood.

A screening procedure is used to effectively test the efficacy of a number of compounds (almost all of which are physiological), that may be used as inhibitors of PK during blood banking. The assay is a modification of the procedure described by Rognstad, R. (Biochem. Biophys. Res. Commun. 63:900-905, 1975). The assay measures the rate of formation of 14C-pyruvate and 14C-lactate from 14C-labelled substrates provided to the red cells which enter glycolysis above the PK step, i.e. it measures the flux at the PK step. The transport of some of these compound is facilitated by using liposome mediated transfer. Successful transfer of high concentrations of L-phenylalanine into intact red cells has been reported by this technique (Kumpathi, J. Biochem. Biophys. Res. Commun. 105:482, 1982).

According to the present invention, the handicaps in glycolysis that may be experienced by the red cell during
storage have been determined and a method has been developed to improve the quality and shelf life of the stored blood.

To improve the quality and prolong the shelf life of blood according to the present invention, the preserving compound(s) is either added to the bag containing the preservative into which blood is drawn directly or is added to the fresh blood or red blood cells immediately after collection into the anticoagulant. Alternatively, standard anticoagulants can be added to the blood immediately upon collection along with the preserving compound(s) of the present invention. The mixture of whole blood or red blood cells, anticoagulant and the preserving compound(s) is then stored at temperatures below 10°C., preferably at a temperature within the range of from about 1°C. to about 6°C or lower with cryopreservative agents, e.g. glycerol.

The whole blood or red blood cells preserved according to the present invention may be stored for longer than 35 days (up to 8-10 weeks and perhaps more), while still being of as good or better quality than blood stored by standard prior art methods, such as with the use of anticoagulant acid citrate-dextrose solutions or anticoagulant citrate-phosphate-dextrose (+adenine) and Adsol solutions.

From the foregoing description and embodiments of the invention, one skilled in the art can make various changes and modifications of the invention to adapt it to various usages and conditions without departing from the spirit and scope of the invention.

What I claim is:
CLAIMS

1. A method for improving the quality and/or increasing the shelf life of whole blood and red blood cell concentrates by manipulating red cell enzymes by adding to the whole blood or red blood cells an effective amount of at least one compound which inhibits the activities of pyruvate kinase and DPGP enzymes and stimulates the activities of FFK, DPGM and PGP at low concentrations, which is capable of permeating red blood cell membrane, and which is physiologically acceptable; and storing said blood.

2. The method of Claim 1 wherein the compound is selected from the group consisting of L-amino acids, free fatty acids, glycolytic intermediates including analogs of phosphoenolpyruvate, free bases, and AMP derivatives and miscellaneous compounds.

3. The method of Claim 2 wherein the L-amino acids are selected from the group consisting of L-alanine, L-phenylalanine, L-cysteine, alpha-amino butyric acid, and their derivatives and structural analogs.

4. The method of claim 2 wherein the free fatty acids are selected from the group consisting of octanoic acid, lauric acid, linoleic acid, oleic acid, palmitic acid, myristic acid, and elaidic acid and their derivatives as well as structural analogs.
5. The method of Claim 2 wherein the glycolytic intermediates and analogs of phosphoenolpyruvate are selected from the group consisting of D-phospholactate, D-phosphopyruvate, phenylpyruvate, and 2- and 3-phosphoglyceric acid phosphoglycolic acid, phosphoglyoxylate, their structural analogs and derivatives.

6. The method of Claim 2 wherein the free bases are selected from the group consisting of adenosine, inosine, cytidine, thymidine, guanosine, and uridine, their derivatives and structural analogs.

7. The method of Claim 2 wherein the AMP derivatives are selected from the group consisting of cAMP, monobutyl-cAMP and dibutyryl cAMP, their structural analogs and derivatives.

8. The method of Claim 2 wherein the miscellaneous compounds are selected from the group consisting of ammonia, glucagon, acetyl Co-A and its derivatives, allantoin, 4-ethylxaloacetate, phenylethylbiguanid and its analogs, and quercetin.

9. The method of Claim 1 wherein the compounds that activate the activities are selected from the group consisting of cobalt sulfate, magnesium chloride, manganese chloride, and nickel sulfate.
10. The method of Claim 1 wherein the inhibitors of ATPP are selected from the group consisting of glycerate -2-P, glycerate -3-P, glyoxylate, AMP, cyclic AMP, Pi, PPI, Malonate, L-lamate, Maleate, D-tartrate, D-tartrate, meso-tartrate, Glutamate, D-L-isocitrate, D-isocitrate, trans-aconitate, cis-aconitate, phyrophosphate, and mixtures thereof.
FIGURE 1

Reactivation studies of diphosphoglycerate mutase using different buffers containing K^+, Pi^-, β-mercaptoethanol and 2,3-DPG as potential activators.
The standard curve for the radio-
mometric assay of glycolate-2-P study;
good linearity between 0.5-6
μL/L concentration.

FIGURE 4

FIGURE 3
INTERNATIONAL SEARCH REPORT

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) 1

According to International Patent Classification (IPC) or to both National Classification and IPC
IPC(4) A61K 35/14 A01N 1/02 A61K 35/18
U.S. 424/101 435/2

II. FIELDS SEARCHED

Classification System Classification Symbols

U.S. 424/101 435/2

Documentation Searched other than Minimum Documentation
to the Extent that such Documents are Included in the Fields Searched 4

CHEMICAL ABSTRACTS VOL. 76 to 104 (1972 to 1986)
"BLOOD PRESERVATION & STORAGE"

III. DOCUMENTS CONSIDERED TO BE RELEVANT 14

<table>
<thead>
<tr>
<th>Category</th>
<th>Citation of Document, 14 with indication, where appropriate, of the relevant passages 17</th>
<th>Relevant to Claim No. 18</th>
</tr>
</thead>
<tbody>
<tr>
<td>X</td>
<td>US, A, 3,874,384 (DEINDOERFER) 1 April 1962. See column 3, line 40, column 4 line 61.</td>
<td>1,6</td>
</tr>
<tr>
<td>X</td>
<td>US, A, 3,925,153 (LABORIT) 9 December 1975. See column 2, line 65 to column 3, line 8.</td>
<td>1</td>
</tr>
<tr>
<td>X</td>
<td>US, A, 4,112,070 (HARMENING) 5 September 1978. See column 2, lines 17-25.</td>
<td>1,6</td>
</tr>
</tbody>
</table>

* Special categories of cited documents: 13
  "A" document defining the general state of the art which is not considered to be of particular relevance
  "E" earlier document but published on or after the international filing date
  "L" document which may throw doubts on priority claim(s) or which is cited to establish thepublication date of another citation or other special reason (as specified)
  "O" document referring to an oral disclosure, use, exhibition or other means
  "P" document published prior to the international filing date but later than the priority date claimed
  "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
  "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step
  "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
  "A" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search 2 23 March 1987
Date of Mailing of this International Search Report 3 31 MAR 1987

International Searching Authority 4

ISA/USA

Signature of Authorized Officer 20

SAM ROSEN

Form PCT/ISA/210 (second sheet) (May 1986)
FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

X Chemical Abstracts Volume 79
No. 1 issued July 9, 1973
(Columbus, Ohio, USA)
KATSADZE et al "Use of salol
and L-aminocaproic acid for the
preservation of blood" see pages
3246, column 2, abstract 3246b

V. OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE

This international search report has not been established in respect of certain claims under Article 17(2) (e) for the following reasons:

1. Claim numbers ..., because they relate to subject matter not required to be searched by this Authority, namely:

2. Claim numbers ..., because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

VI. OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING

This International Searching Authority found multiple inventions in this International application as follows:

1. As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.

2. As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3. No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4. As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

- The additional search fees were accompanied by applicant's protest.
- No protest accompanied the payment of additional search fees.

Form PCT/ISA/210 (supplemental sheet (2) (May 1986)
<table>
<thead>
<tr>
<th>Category</th>
<th>Citation of Document, with indication, where appropriate, of the relevant passages</th>
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</thead>
<tbody>
<tr>
<td>X</td>
<td>Chemical Abstracts Vol. 92, No. 21 issued May 26, 1986 (Columbus, Ohio, USA) ROMERO et al &quot;In vitro restoration of blood preserved in CPD and CPD-adenine by adding inosine and pyruvate phosphonate see page 398, column 2 abstract 178, 188e</td>
<td>1,5</td>
</tr>
<tr>
<td>L</td>
<td>Chemical Abstracts Vol. 104 No. 18 issued May 5, 1986 (Columbus, Ohio, USA) SATAKE et al &quot;Preservatives for blood transfusion&quot; see page 457, column 1, abstract 156053w</td>
<td>1,2</td>
</tr>
</tbody>
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