The Respiratory Function of Blood:
Transfusion and Blood Storage

Rita McConn, Ph.D.,* and John B. Derrick, Ph.D.†

Mean $P_{50}$ and 2,3-DPG in eight trauma patients receiving massive transfusions (average 33 units) of stored ACD blood were 19.18 mm Hg and 0.24 mol/mol Hb, respectively. These values are markedly below those of healthy non-smoking control subjects ($P_{50}$ 25.2 mm Hg, 2,3-DPG 0.76 mol/mol Hb), which were not attained in the patients until four days after transfusion. There was a direct correlation between $P_{50}$ and 2,3-DPG. The progressive decreases in $P_{50}$ and 2,3-DPG which occurs in erythrocytes stored in ACD were prevented by pre-storage supplementation with adenine (1 mol) and inosine (10 mol). These additives, however, significantly increased the rate of flux of cellular sodium and potassium. In contrast, preservation of erythrocytes using a low-glycerol-rapid-freeze technique maintained $P_{50}$ at pre-freeze values despite a 27 per cent loss of 2,3-DPG, with no appreciable effect on ionic flux and ATP concentration, indicating that erythrocytic respiratory function is best maintained by storage by freeze-preservation when freshly drawn.

(Key words: 2,3-DPG; Oxygen affinity of hemoglobin; Stored blood; Adenine–inosine supplementation; Ionic flux in erythrocytes; Frozen erythrocytes.)

Until very recently, methods of storing blood were assessed on the basis of effects on viability of preserved erythrocytes rather than function, yet the primary purpose of transfusing whole blood is to maintain the delivery of oxygen to the tissues. There are at present no in-vitro measurements which specifically indicate the efficiency of erythrocytic respiratory function. Assessment can be made in vitro, however, through determination of the oxygen dissociation curve of whole blood. The position of the curve is, for comparative purposes, usually defined by the partial pressure of oxygen necessary to produce half-saturation of hemoglobin (i.e., when reduced hemoglobin concentration equals oxyhemoglobin concentration) at constant pH and temperature, and it is commonly expressed as $P_{50}$. For normal man the value is 26.52 at pH 7.40 (the intracellular pH when plasma pH is 7.4) and 37.5 C. A decrease in the affinity of cellular hemoglobin for oxygen shifts the dissociation curve to the right of normal and is denoted by a higher $P_{50}$, whereas an increase in oxygen affinity results in the reverse situation, that is, a shift of the curve to the left and a $P_{50}$ lower than normal.

Recently, it has been shown that the affinity of hemoglobin for oxygen is affected by the erythrocytic content of 2,3-diphosphoglyceric acid (2,3-DPG) and adenosine triphosphate (ATP). Figure 1 shows the positions of these compounds in the metabolic pathways of the erythrocyte. It seems likely that, of the two compounds, 2,3-DPG has the greater effect on the affinity of hemoglobin for oxygen, since its concentration in the erythrocyte is approximately four times that of ATP.

Early biochemical studies of blood showed rapid progressive decreases in cellular 2,3-DPG during storage at acidic pH. The progressive shift to the left of the oxygen dissociation curve of blood stored in acid citrate dextrose (ACD) at 4 C was first reported by Valtis and Kennedy in 1950. They also showed that the oxygen dissociation curves of recipients of stored blood were shifted to the left. The degree of shift in the transfused patient was proportional to the amount and age of transfused blood, and recovery of the curve to normal took several hours to several days. Addition of sodium chloride to the stored citrated blood to give a final concentration of 0.4–0.5 per cent corrected the abnormality of oxygen dissociation in vitro and in vivo, and therefore these investigators ascribed the shift to altered electrolytic and osmotic relationships between

* Assistant Professor of Surgery, Albert Einstein College of Medicine, Bronx, New York 10461.
† Associate Investigator, New York Blood Center, New York, New York 10021.
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the erythrocytes and plasma of both stored and transfused blood. Seventeen years later, the Benesches called 2,3-DPG “a super salt,” since they found that its effect on the affinity of hemoglobin for oxygen was similar to that of inorganic salts except that it was effective in much lower concentrations. Thus, the Valtis–Kennedy effect appears to be related to the changes in 2,3-DPG concentration in ACD blood during storage. We have examined this hypothesis in a study of trauma patients receiving massive transfusions of stored ACD blood, and the results are contained in this report.

We have also studied the affinity for oxygen of blood kept under two sets of storage conditions which seemed likely to delay depletion of the 2,3-DPG and ATP of the erythrocyte. The first was pre-storage addition of adenine and/or inosine. Adenine supplies the purine base, which the erythrocyte cannot synthesize, and inosine provides ribose, which can be used as a substrate for the production of ATP and 2,3-DPG via the pentose monophosphate shunt and the triose phosphate portion of the glycolytic sequence (fig. 1). Addition of adenosine alone would achieve the same purpose (fig. 1), but supplementation with the two compounds separately and together enabled differentiation between the metabolic effects of the purine and the sugar. The second method studied was freeze-preservation, since previous work had already shown that this procedure has no effect on the concentration of ATP.
Materials and Methods

Eight trauma patients who received massive transfusions of ACD blood (average 33 units) were studied. The hospital blood bank was not instructed to provide ACD blood stored for any specific period; the transfused blood had been stored for various periods, none longer than 20 days. The patients were 18 to 35 years old and had been healthy prior to injury. Six patients had fractures of the pelvis and/or lower limbs; the remaining two had sustained abdominal gunshot wounds. Immediately after the completion of transfusion blood samples were drawn into heparinized syringes and analyzed for $P_{50}$ and 2,3-DPG. Thereafter, samples were drawn daily until values in the range of those of healthy controls were reached and maintained.

Experiments to study the effects of pre-storage addition of adenine and/or inosine to ACD blood were set up as follows:

A unit of blood was collected to ACD and divided into four aliquots. One served as a control, and to each of the remaining three one of the following was added: adenine (1 mmol), inosine (10 mmol), or adenine (1 mmol) plus inosine (10 mmol) before the blood was stored at 4 C. Six units of blood were treated in this way. Two of these were stored for 15 days and analyzed on alternate days, while the remaining four were stored and analyzed weekly during a period of seven weeks. The following were measured before and during storage: $P_{50}$, 2,3-DPG, ATP, intracellular electrolyte concentrations were determined by a modification of the method of Funder and Wieth.

Results

The following values (mean ±SD) were established in our laboratory for heparinized blood from 17 non-smoking volunteers: $P_{50}$ 25.2 ± 1.1 mm Hg; 2,3-DPG 0.76 ± 0.16 mol/mol Hb; ATP 3.65 ± 0.73 mmol/g Hb; sodium 14.0 ± 3.7 mEq/kg and potassium 82.1 ± 4.6 mEq/kg erythrocytes.

Mean $P_{50}$ and 2,3-DPG in the trauma patients receiving massive transfusions are shown in table 1. Initial values for both on completion of transfusion were markedly below those established for healthy non-smoking control subjects. These levels were not attained in the patients until about four days later. They were subsequently maintained throughout the recovery period. Throughout there was a direct correlation between $P_{50}$ and 2,3-DPG.

The pH of freshly-drawn ACD blood was approximately 7.10. $P_{50}$ decreased progressively during storage at 4 C, falling to 6.6 after 21 days and 6.5 after 50 days. Essentially similar pH values were found in the samples supplemented with adenine and/or inosine.

The results of the storage experiments on ACD blood preserved at 4 C with and without additives are presented in figures 2 and 3. Figure 2 shows that $P_{50}$ of the blood when freshly collected in ACD was 21.59 ± 2.35 mm

<table>
<thead>
<tr>
<th>Day</th>
<th>$P_{50}$ (mm Hg) Mean ± SD</th>
<th>2,3-DPG (N/MM Hb) Mean ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 1</td>
<td>19.18 ± 1.49</td>
<td>0.244 ± 0.13</td>
</tr>
<tr>
<td>Day 2</td>
<td>21.02 ± 3.90</td>
<td>0.390 ± 0.22</td>
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<tr>
<td>Day 3</td>
<td>23.03 ± 0.60</td>
<td>0.536 ± 0.07</td>
</tr>
<tr>
<td>Day 4</td>
<td>25.13 ± 0.13</td>
<td>0.78 ± 0.12</td>
</tr>
</tbody>
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Hg (mean ±SD). Storage at 4 C resulted in a decrease of $P_{50}$ to approximately 14.6 mm Hg after 10 days for both the control ACD blood and that supplemented with adenine. The $P_{50}$ of the latter remained constant for the remainder of the observation period, while the $P_{50}$ of the control decreased a further 2 mm Hg.

Samples containing inosine alone were found to have $P_{50}$ values at or above initial levels for 14 days, but after 50 days of storage the values had fallen to the same levels as those of samples containing adenine. However, the addition of adenine together with inosine maintained $P_{50}$ at or above normal throughout the 50-day observation period. The trends apparent in the $P_{50}$ studies are reflected in the values for erythrocytic 2,3-DPG, with the exception of the samples containing inosine alone, in which the decrease in 2,3-DPG was not as pronounced as the decrease in $P_{50}$.

Differences in the effects of the various prestorage additives on ATP concentration did not become significant until after 30 days of storage at 4 C (fig. 2). It then became apparent that the adenine-inosine combination is the most effective in maintaining ATP at or above the minimum level needed for viability (ca. 2.3 mmol ATP/g Hb) until the end of the 50-day observation period.

The initial intracellular sodium and potassium values of fresh ACD blood were 13.2 ± 1.5 mEq/kg (mean ±SD) and 83.0 ± 3.8 mEq/kg erythrocytes, respectively. As would be expected, storage at 4 C caused a progressive decrease in cellular potassium and an in-

![Fig. 2. Mean $P_{50}$, 2,3-DPG, and ATP values in ACD blood stored at 4 C with and without prestorage addition of adenine and/or inosine (n = 6).](image-url)
increase in cellular sodium in every sample (fig. 3). The rate of ionic flux, however, showed a reverse distribution of the response to the additives to that seen in P_{50} and 2,3-DPG, i.e., the greatest decreases in cellular potassium and increases in cellular sodium occurred in the presence of adenine and inosine. Calculation of the intracellular ionic concentration, i.e., sodium plus potassium concentration, showed a progressive decrease in this value in every sample during storage, and again the same distribution of response to additive was seen, such that samples containing adenine and inosine after 50 days had an intracellular ionic concentration of 80 mEq/l compared with 86 mEq/l in the control samples and in those supplemented with adenine. These changes in electrolyte concentration were not the result of losses of intracellular water.\\textsuperscript{14}

The results of freeze-preservation experiments are shown in histogram form in figures 4 and 5. Values in the blood before freezing showed a response to storage in ACD similar to that observed in the previous experiment, i.e., progressive decreases in P_{50} and 2,3-DPG and the same pattern of electrolyte movement. Analysis of blood samples immediately before freezing and again after thawing and reconstitution showed very little change in P_{50}, i.e., 1–2 mm Hg, although 2,3-DPG was found to have fallen an average of 26.8 per cent. The magnitudes of 2,3-DPG losses were consistent despite the fact that the actual values varied according to the periods of storage in ACD at 4 C before freeze-preservation. ATP concentration remained unaffected by the freeze process. The sodium content of the freeze-preserved erythrocytes increased an average of 10
per cent, with no significant change in intracellular potassium.

The interrelationship between \( P_{50} \) and 2,3-DPG found in these studies is shown in figure 6. In stored ACD blood the relationship between these values is curvilinear, and interpolation of the graph plot shows that when 2,3-DPG is zero, \( P_{50} \) is 11.3 mm Hg. In transfused blood the relationship between \( P_{50} \) and 2,3-DPG is linear; when 2,3-DPG is zero, \( P_{50} \) is 16.3 mm Hg. The data also indicate that an increase in 2,3-DPG of 0.16 mol/mol Hb in the blood of the transfused patient is accompanied by an increase in \( P_{50} \) of 1 mm Hg.

**Discussion**

The graphic representation of the collective data from this study (fig. 6) shows that the relationship between 2,3-DPG and the affinity of hemoglobin for oxygen (measured by \( P_{50} \)) which was established by the studies of the Benesch's for hemoglobin solutions also holds for both the transfused circulating erythrocyte and the stored erythrocyte. The results indicate that the relationship is influenced by the erythrocytic environment. Transfusion of ACD blood results in conversion from a curvilinear to a straight-line relationship, indicating the influence of environmental change.

\( P_{50} \) and 2,3-DPG values after massive transfusion in trauma patients fell in the same range as those in ACD blood stored at 4 C for one to two weeks. Browne and co-workers also found similar changes in the blood of patients receiving massive transfusions of ACD blood or frozen erythrocytes (stored five to six days in ACD before freezing). In some of their patients the shift to the left after transfusion was followed by a shift to the right before the dissociation curve returned to normal.
They, too, found a correlation between $P_{50}$ and 2,3-DPG in every patient. While our results and those of Broennle et al. could be interpreted as indicative that the Valtis–Kennedy effect is the result of changes in 2,3-DPG in stored blood used for transfusion, it would be unwise not to take into account the effects on the oxygen dissociation curve of the metabolic response to trauma or disease. Such an evaluation is not feasible at present, since the interrelationship between the various physicochemical factors which affect the dissociation curve in acute illness are not clearly understood.16

The marked difference between the $P_{50}$ of freshly-drawn ACD blood and that of freshly-drawn heparinized blood from normal non-
smoking individuals cannot be attributed to the effect of 2,3-DPG, since there was essentially no difference between cellular concentrations of this metabolite. Current studies in this laboratory indicate that an explanation may lie in the fact that the Severinghaus correction factors used in this study to convert \( P_{50} \) of ACD blood to \( \text{pH} \) 7.4 assume a normal \( \text{pH} \) gradient across the erythrocyte, and we have found that the citrate ion lowers this gradient to approximately 0.05. The \( \text{pH} \) determining the affinity of hemoglobin for oxygen in whole blood is the intracellular \( \text{pH} \). Recalculation of \( P_{50} \) of ACD blood to the intracellular \( \text{pH} \) of heparinized blood, while modifying the position of the curvilinear relationship between \( P_{50} \) and 2,3-DPG (fig. 6), does not eliminate the difference between this curve and the linear relationship in heparinized blood.

Many years ago, Guest and Rapoport\(^4\) showed a rapid breakdown of 2,3-DPG in the blood when \( \text{pH} \) approaches 7.0. The initial \( \text{pH} \) of blood drawn into ACD is approximately 7.0, and it decreases to 6.7 after three weeks of storage at 4 C. Thus, the effect of \( \text{pH} \) on 2,3-DPC becomes significant in terms of the effect of storage of the erythrocyte in ACD. The studies of Minakami\(^1\) suggest that the decrease in 2,3-DPG in an acidic \( \text{pH} \) results from a reduction in the rate of synthesis resulting from the inhibition of phosphofructokinase (catalyzes the reaction fructose 6-P \( \rightarrow \) fructose 1:6 di-P) in this \( \text{pH} \) range.

Supporting evidence for this concept was apparent in this study when inosine was added to ACD blood either alone or with adenine. In figure 1 it is evident that this nucleoside can be utilized by the pentose shunt and enter the glycolytic pathway as triose phosphate, thereby circumventing the phosphofructokinase reaction. This allows maintenance of 2,3-DPG levels, and thus of \( P_{50} \), for longer than in non-supplemented blood.

The results indicate that the favorable effects of adenine and inosine supplementation of ACD blood occur at the cost of an increase in the ionic flux. Changes in \( \text{pH} \) are known to affect ionic movement across the erythrocytic membrane.\(^1\) However, all samples with and without additives had the same initial \( \text{pH} \) and essentially the same \( \text{pH} \) decrease during storage; therefore, any possibility that this might be the causative factor can be dismissed. The overall decreases in ionic concentrations varied according to additive used, so the effect must be more than a matter of simple inhibition of active processes by low temperature.

The data show that ionic movement was greatest in those samples with the highest 2,3-DPG concentrations. This supports the possibility of an interrelationship between the concentration of this organic phosphate and the ionic gradient of the erythrocyte, as suggested many years ago by Rapoport and Guest\(^1\) and more recently by Gardos.\(^5\)

In contrast to adenine–inosine supplementation of ACD blood, freeze-preservation of erythrocytes maintained \( P_{50} \) values at pre-freeze levels without a marked effect on the ionic flux and in spite of a 27 per cent decrease in 2,3-DPG. Subsequent studies have in-
The obvious aim in preserving blood is to maintain the structure and function of the erythrocyte throughout storage, the longer the better. While the pre-storage addition of inosine, enhanced by the presence of adenine, does maintain or even improves respiratory function of the erythrocyte in ACD, this occurs at the expense of ionic gradient. Hence, transfusion of more than a few units of inosine-supplemented blood might, in addition to imposing on the recipient an increased uric-acid load from the metabolism of the nucleoside, also cause hypokalemia. It appears that, for the present, both metabolic integrity and respiratory function of erythrocytes can be best maintained during storage by freeze-preservation when they are freshly drawn.

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References
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