The In Vitro Restoration of Red Cell 2,3-Diphosphoglycerate Levels in Banked Blood

By Frank A. Oski, Susan F. Travis, Leonard D. Miller, Maria Delivoria-Papadopoulos, and Elizabeth Cannon

The demonstration that red cell levels of 2,3-DPG play a central role in determining the affinity of hemoglobin for oxygen has resulted in a renewed interest in methods for maintaining or restoring the level of this organic phosphate in stored blood. The effects of addition of inorganic phosphate, inosine and pyruvate, individually or in various combinations, all in a final concentration of 10 mM were evaluated 1 and 4 hours after supplementation of ACD-stored blood, 21 to 28 days old. In 14 studies the initial 2,3-DPG level averaged 176 μmoles/ml RBC. In normal fresh blood the 2,3-DPG was 4200 ± 400 μmoles/ml RBC. Inosine addition raised the 2,3-DPG to 1395, inosine and phosphate to 1528, inosine and pyruvate to 3363, while the combination of inosine, pyruvate and phosphate increased the level to 6637 μmoles/ml RBC. After 2-3 hours of incubation most of the 2,3-DPG restoration had occurred. The maximum effects did not require prior pH correction of the blood. Although inosine in a final concentration of 10 mM was required for optimum effects, the phosphate and pyruvate concentrations could be reduced to 4 mM. In the presence of inosine and phosphate alone the red cell accumulated large quantities of triose phosphates, fructose diphosphate and glucose-6-phosphate. These levels were reduced by the addition of pyruvate. Pyruvate addition appears necessary to provide sufficient NAD for maximum 2,3-DPG synthesis. Associated with 2,3-DPG restoration of the stored blood there was a rise in the P50 (the oxygen tension at which hemoglobin is 50% saturated) from a mean of 16.7 to 31.6 mm. Hg.

Since 1967 when Benesch and Benesch1 and Chanutin and Curnish2 demonstrated that the affinity of a hemoglobin solution for oxygen may be

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decreased by its interaction with organic phosphates, the role of these com-
ponents in altering the oxygen-hemoglobin dissociation curve of the intact
human erythrocyte has been a subject of intense investigation. The principal
organic phosphate of the human erythrocyte is 2,3-diphosphoglycerate (2,3-
DPG) and its content within the cell has been shown to correlate precisely
with the position of the oxygen-hemoglobin dissociation curve, as reflected
by the P$_{50}$ (the partial pressure of oxygen at which hemoglobin is 50% satu-
rated) in a variety of clinical conditions which include hypoxic states such
as exposure to high altitude$^3$ and cyanotic heart disease,$^4$ a variety of anemias,$^5$
hyperthyroidism,$^6$ septic shock,$^7$ and the changes associated with blood
storage.$^8$

In blood stored under conventional blood bank conditions the 2,3-DPG
level drops sharply and by 10 days of storage 2,3-DPG levels are only 20–25
per cent of their original level and by 21 days of storage they have fallen to
10 per cent of their initial content.$^8$ The addition of inosine to 2,3-DPG
poor blood has been found to result in a partial restoration of 2,3-DPG
levels and a decrease in the affinity of hemoglobin for oxygen.$^{10,11}$ The purpose
of this communication is to report a technique for restoring 2,3-DPG levels
to normal or supranormal levels and examine the mechanism by which this
result is achieved.

METHODS

Blood stored for periods of 21–28 days at 4°C in sterile plastic bags (Fenwal Labora-
tories, Morton Grove, Ill.) containing acid-citrate-dextrose (NIH Formula A) was utilized
for all the studies described.

Blood was removed from the bags and incubated for 4 hours at 37°C in 25 ml. flasks
in a metabolic shaker oscillating at 80 cycles per minute. The blood was supplemented
with varying combinations and concentrations of inosine (Calbiochem), inorganic phosphate
(disodium hydrogen phosphate) and sodium pyruvate (Calbiochem). In some experiments
the blood was divided into two large portions before further supplementation. One of the
samples was buffered to pH 7.4 by the addition of 1.2 M Tris (hydroxymethyl aminomethane).
This usually required the addition of 1.0 to 1.5 ml. of Tris per 50 ml. of blood.
At the start of the incubation, and at periodic intervals during the 4-hour incubation, 2 ml.
portions of the blood were removed and added to 4 ml. of cold 2 N perchloric acid for
the extraction of glycolytic intermediates. At each sampling period, microhematocrit de-
terminations were performed. The perchloric acid treated samples were processed and
neutralized as previously described$^{12}$ and then assayed for 2,3-diphosphoglycerate,$^{13}$
glucose-6-phosphate, fructose-6-phosphate,$^{13}$ triose phosphates,$^{14}$ and adenosine triphos-
phate.$^{13}$

The P$_{50}$, the oxygen tension in mm. Hg at which hemoglobin is 50 per cent saturated
was determined by methods previously described.$^4$

RESULTS

In the initial studies inosine, pyruvate, and inorganic phosphate were all
studied at a final concentration of 10 mM. In 14 experiments, the initial red
cell 2,3-DPG averaged 176 m$_{m}$moles/ml. RBC. After 4 hours incubation
in the presence of inosine alone, the 2,3-DPG rose to 1395 m$_{m}$moles/ml. RBC
(Table 1). The combination of inosine and phosphate had no significant
additive effect. When inosine and pyruvate were added in combination, the
Table 1.—2, 3-Diphosphoglycerate (2, 3-DPG) Synthesis in Stored Blood Incubated With Combinations of Inosine, Pyruvate and Inorganic Phosphate

<table>
<thead>
<tr>
<th>Additive</th>
<th>Final Concentration (mM)</th>
<th>0 time</th>
<th>1 hour</th>
<th>4 hour</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inosine (14)*</td>
<td>10</td>
<td>176±178</td>
<td>868±456</td>
<td>1395±577</td>
</tr>
<tr>
<td>Inosine and inorganic phosphate (14)</td>
<td>10</td>
<td>176±178</td>
<td>874±421</td>
<td>1528±458</td>
</tr>
<tr>
<td>Inosine and pyruvate (14)</td>
<td>10</td>
<td>176±178</td>
<td>2593±783</td>
<td>3365±1418</td>
</tr>
<tr>
<td>Inosine, pyruvate and inorganic phosphate (14)</td>
<td>10</td>
<td>176±178</td>
<td>4579±1217</td>
<td>6637±1433</td>
</tr>
</tbody>
</table>

* Number in parenthesis refers to number of experimental studies.

2,3-DPG level rose to 3363 μmoles/ml. RBC at the end of 4 hours, while the combination of inosine-pyruvate and inorganic phosphate produced a dramatic rise to 6637 μmoles/ml. RBC. Incubation in the absence of additives or with pyruvate or phosphate alone produced no increase in 2,3-DPG. The normal red cell 2,3-DPG in this laboratory, mean of 40 subjects, is 4200 ± 400 μmoles/ml. RBC.

In a second series of five experiments samples were examined at hourly intervals during the incubation. The major increase in 2,3-DPG levels occurred during the first 2 hours of incubation reaching a mean value of 4510 μmoles/ml. RBC (Fig. 1). This represented 73.7 per cent of the final mean value of 6100 μmoles/ml. RBC.

In a third series of experiments, the level of two of the three additives was held constant at 10 mM and the third additive was varied from 0 to 10 mM. These studies (Table 2) demonstrated that pyruvate and inorganic phosphate supplements at a final concentration of 4 mM gave near optimal results, while the effect of inosine was dose-dependent throughout the range tested. When inosine was combined with pyruvate and inorganic phosphate, both at a final concentration of 4 mM, the effects on 2,3-DPG synthesis were as great as when all three were present in a final concentration of 10 mM (Table 3).

Fig. 1.—Rate of 2,3-DPG restoration with time of incubation in the presence of inosine, pyruvate and phosphate, all in a final concentration of 10 mM.
Table 2.—Effect of Varying the Concentration of Inosine, Pyruvate or Inorganic Phosphate While the Other Two are Held Constant at 10 mM*

<table>
<thead>
<tr>
<th>Concentration of Variable mM</th>
<th>2, 3-DPG at 4 (mMoles/ml. RBC)</th>
<th>Variable</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pyruvate</td>
<td>Inorganic Phosphate</td>
</tr>
<tr>
<td>0</td>
<td>1376</td>
<td>2349</td>
</tr>
<tr>
<td>2</td>
<td>4969</td>
<td>4022</td>
</tr>
<tr>
<td>4</td>
<td>7103</td>
<td>5631</td>
</tr>
<tr>
<td>6</td>
<td>7340</td>
<td>5709</td>
</tr>
<tr>
<td>10</td>
<td>7393</td>
<td>6203</td>
</tr>
</tbody>
</table>

* Average of three experiments.

Table 3.—Comparison of All Three Additives at 10 mM with Inosine at 10 mM and Pyruvate and Inorganic Phosphate at 4 mM*

<table>
<thead>
<tr>
<th>Time</th>
<th>2,3-DPG (mMoles/ml. RBC)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>354±105</td>
</tr>
<tr>
<td>1</td>
<td>3262±420</td>
</tr>
<tr>
<td>4</td>
<td>6160±720</td>
</tr>
</tbody>
</table>

* Mean of three experiments.

When the pattern on glycolytic intermediates was examined it was found that in the absence of pyruvate there was a marked accumulation of the triose phosphates and fructose-diphosphate (Table 4). Adenosine triphosphate levels returned to normal with and without pyruvate, although somewhat higher values were observed in its presence. The prior buffering of the blood to pH 7.4 before supplementation with the additives did not increase the magnitude of the 2,3-DPG resynthesis. On three occasions blood was transferred and incubated anaerobically in plastic bags. The rise in 2, 3-DPG was identical in this closed system to that observed in simultaneous studies performed in open flasks. The rate of 2,3-DPG synthesis was evaluated at 4°C, 25°C and 37°C after a 4-hour period of incubation. The 2,3-DPG synthesis at 4°C was negligible, representing an average of 6.2 per cent of that observed.

Table 4.—Pattern of Glycolytic Intermediates in Blood Incubated With Inosine (10 mM) and Inorganic Phosphate (4 mM) With and Without Added Pyruvate (4 mM*)

<table>
<thead>
<tr>
<th></th>
<th>Without Added Pyruvate</th>
<th>With Added Pyruvate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mMoles/ml. RBC</td>
<td></td>
</tr>
<tr>
<td>Glucose-6-phosphate</td>
<td>137.0</td>
<td>282.0</td>
</tr>
<tr>
<td>Fructose-6-phosphate</td>
<td>27.5</td>
<td>67.6</td>
</tr>
<tr>
<td>Triose phosphates (Glyceraldehyde-3-phosphate, dihydroxyacetone phosphate) and fructose diphosphate</td>
<td>8503.0</td>
<td>84.0</td>
</tr>
<tr>
<td>2, 3-diphosphoglycerate</td>
<td>1286.0</td>
<td>4014.0</td>
</tr>
<tr>
<td>Adenosine triphosphate</td>
<td>1011.0</td>
<td>1261.0</td>
</tr>
</tbody>
</table>

* At end of 4 hours of incubation (mean of three experiments).
Table 5.—The Effects of 2, 3-DPG Restoration on the $P_{50}^*$ of Stored Blood

<table>
<thead>
<tr>
<th></th>
<th>Before Supplementation</th>
<th>After</th>
</tr>
</thead>
<tbody>
<tr>
<td>$P_{50}$ (mm. Hg)</td>
<td>16.7 (14.5–18.6)</td>
<td>31.6 (28.6–37.0)</td>
</tr>
<tr>
<td>2, 3-DPG (molecules/ml.)</td>
<td>210 (0–450)</td>
<td>6800 (5476–10,961)</td>
</tr>
</tbody>
</table>

$^*$At pH 7.4, temperature 37.0°C.

at 37°C. At 25°C the 2,3-DPG levels averaged 51 per cent of those achieved at 37°C.

In ten additional experiments the $P_{50}$, at 37°C, pH 7.40, was determined before and after a 4-hour incubation of blood with supplemental inosine (10 mM), pyruvate (4 mM) and inorganic phosphate (4 mM). The mean $P_{50}$ prior to 2,3-DPG restoration was 16.7 mm. Hg (Table 5). After a 4-hour incubation, the $P_{50}$ had risen to a mean value of 31.6 mm. Hg. The $P_{50}$ for 20 normal, nonsmoking adult males, as determined in our laboratory is $27.0 \pm 1.1$ mm. Hg.

**Discussion**

The addition of inosine to stored blood is believed to result in 2,3-DPG synthesis because it is acted on by red cell nucleoside phosphorylase to yield ribose-1-phosphate and hypoxanthine$^{15}$ (Fig. 2). Inorganic phosphate serves as a necessary substrate in this process of phosphorolysis. The ribose-1-phosphate formed is then isomerized to ribose-5-phosphate, enters the pentose phosphate pathway and is eventually recycled back to either fructose-6-phosphate or glyceraldehyde-3-phosphate. The glyceraldehyde-3-phosphate generated is then converted to 1,3-diphosphoglycerate in the presence of

Fig. 2.—The pathway of red cell inosine metabolism to 2,3-DPG. Abbreviations: PFK, phosphofructokinase; G-3-PD, glyceraldehyde-3-phosphate dehydrogenase; PGK, phosphoglycerate kinase; DPGM, diphosphoglycerate mutase; DPGP, diphosphoglycerate phosphatase.
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Glyceraldehyde-3-phosphate dehydrogenase and NAD. The 1,3-diphosphoglycerate is subsequently converted to either 2,3-diphosphoglycerate or 3-phosphoglycerate. The conversion of glyceraldehyde-3-phosphate to 1,3-diphosphoglycerate is limited by NAD availability. If 2,3-DPG accumulates then insufficient amounts of 1,3-diphosphoglycerate are metabolized to pyruvate and lactate with regeneration of NAD from the accumulated NADH. The provision of exogenous pyruvate allows for continued NAD availability and continued DPG synthesis. These observations support the original hypothesis of Borgese and McManus that NAD availability limits red cell inosine metabolism.

When patients receive large quantities of stored blood poor in 2,3-DPG it has been demonstrated that the oxygen-hemoglobin dissociation curve of their blood shifts to the left. In vivo 2,3-DPG reconstitution begins to occur immediately and although as much as 75 per cent of the normal level may be reached in 6 hours, it may take several days for the oxygen dissociation curve and the 2,3-DPG level to return completely to their initial values. During this interval the patient's ability to extract oxygen from his circulating red cells is compromised. In situations of profound anemia where oxygen delivery to the tissues is critical, transfusions of blood rich in DPG would be desirable. It would appear that the simple process of the addition of inosine, pyruvate and inorganic phosphate can make stored blood immediately more functional. The concentration of inorganic phosphate required is very similar to the concentration of inorganic phosphate now present in citrate-phosphate-dextrose (3.7 mM) anticoagulant and this has been used without any apparent deleterious consequences of hyperphosphatemia. The pyruvate added should be quickly metabolized and would not prove to be clinically disadvantageous.

The addition of pyruvate appears to be the extra additive that makes this process of 2,3-DPG restoration superior to those previously reported. The toxic effects of this combination and its degradation products, hypoxanthine and uric acid, as well as the potential bacterial hazards of blood warming must be evaluated before clinical trials can be recommended.

REFERENCES


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