Effect of Oxalate and Malonate on Red Cell Metabolism

By Ernest Beutler, Linda Forman, and Carol West

The addition of oxalate to blood stored in Citrate-phosphate-dextrose (CPD) produces a marked improvement in 2,3-diphosphoglycerate (2,3-DPG) preservation; an increase in 2,3-DPG levels can also be documented in short-term incubation studies. Oxalate is a potent in vitro inhibitor of red cell lactate dehydrogenase, monophosphoglycerate mutase, and pyruvate kinase (PK). In the presence of fructose 1,6-diphosphate the latter inhibitory effect is competitive with phosphoenolpyruvate (PEP). Determination of the levels of intermediate compounds in red cells incubated with oxalate suggest the presence of inhibition at the PK step, indicating that this is the site of oxalate action. Apparent inhibition at the glyceraldehyde phosphate dehydrogenase step is apparently due to an increase in the NADH/NAD ratio. Oxalate had no effect on the in vivo viability of rabbit red cells stored in CPD preservatives for 21 days. Greater understanding of the toxicity of oxalate is required before it can be considered suitable as a component of preservative media. However, malonate, the 3-carbon dicarboxylic acid analogue of oxalate, did not inhibit pyruvate kinase nor affect 2,3-DPG levels.

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VER A DECADE AGO it was demonstrated that ascorbic acid exerted a favorable effect on the 2,3-diphosphoglycerate (2,3-DPG) level of erythrocytes in stored blood.1,2 Subsequent studies revealed that ascorbic phosphate, a more stable derivative of ascorbic acid had a similar effect.3 Although this phenomenon has been confirmed repeatedly,4,5 our investigation of the mechanism of the ascorbate effect failed to provide an explanation for the increase in 2,3-DPG that occurred.6,7 Specifically, we could not find significant inhibition of glycolytic enzymes by ascorbate,8 no evidence of oxidation of either NADH or NADPH, and no evidence that the ascorbate was metabolized or that it exerted a significant effect on intracellular pH.9

Recently, Kandler et al10 found that in contrast to the usual preparations, highly purified ascorbic acid and ascorbic phosphate had no effect on the 2,3-DPG of stored red cells. Searching for a contaminant that might explain the effect of less purified batches, they discovered that oxalate found in the ascorbate preparations could account for the effect on the 2,3-DPG levels of red cells in stored blood.

We now report on the effects of oxalate on the metabolism of human erythrocytes, identifying some of the inhibitory actions that may explain the effect of this compound on 2,3-DPG levels. We have also explored the effect of malonic acid, a natural metabolic intermediate and the 3-carbon analogue of the 2-carbon dicarboxylic acid, oxalic acid, on 2,3-DPG levels.

MATERIALS AND METHODS

After obtaining informed consent, blood samples from normal blood donors were drawn into citrate-phosphate-dextrose (CPD), for storage studies at 4°C or into 1 mg/mL ethylenediamine tetracetic acid (EDTA) for short-term incubations at 37°C. Potassium oxalate and sodium malonate were purchased from Sigma Chemical Company, St. Louis.

Unless otherwise indicated red cell enzyme activities were measured using standard methods,11 adding the stated amount of oxalate to the assay system. Pyruvate kinase (PK) activity was estimated using a system in which the generation of adenosine triphosphate (ATP) is measured in order to circumvent the inhibitory effect of oxalate on the lactate dehydrogenase reaction used as an indicator in the standard method. In this technique, the reaction mixture contained the following: 100 mmol/L Tris-HCl, pH 8; 0.5 mmol/L EDTA; 10 mmol/L MgCl2; 100 mmol/L KCl; 0.2 mmol/L NADP; 2 mmol/L glucose; 1.5 mmol/L adenosine diphosphate (ADP); 0.1 U each of glucose-6-phosphate (G-6-P) dehydrogenase and hexokinase. The reaction was started by the addition of phosphoenolpyruvate (PEP) to provide a concentration of 5 mmol/L. The effect of oxalate on the kinetics of the PK reaction was studied using partially purified PK.12 Diphosphoglycerate phosphatase (DPGP) activity was measured by estimating inorganic phosphate (P) release from labeled 2,3-DPG.13 ATP, 2,3-DPG, and pyruvate and lactate levels were measured spectrophotometrically as previously described.14 G-6-P, fructose-6-phosphate (F-6-P), fructose-1,6-diphosphate (FDP), dihydroxyacetone phosphate (DHAP), glyceraldehyde-3-phosphate (GAP), 3-phosphoglyceric acid (3-PGA), 2-phosphoglyceric acid (2-PGA), and PEP were all measured fluorometrically.15 In some instances, the levels of DHAP, GAP, and FDP were sufficiently elevated in the erythrocytes so that their levels could also be measured by a spectrophotometric modification of the standard fluorometric technique. The internal pH of stored red cells was measured at 4°C using a Corning model 150 with a glass electrode, packing the erythrocytes by centrifugation, freezing, and thawing. Red cell viability was studied in New Zealand white rabbits using the combined 51Cr method.16

RESULTS

Cold storage of CPD anticoagulated blood with and without oxalate. Four hundred fifty milliliters of blood

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from each of four normal donors was collected into 63 mL of CPD and into CPD containing sufficient potassium oxalate to provide a final concentration of 500 μmol/L. The blood was stored at 4°C, mixed at intervals, and the levels of ATP and 2,3-DPG were determined. The results of these investigations are shown in Fig 1. There was no difference in internal red cell pH for the first 2 weeks of storage. At 3 weeks the internal pH of the samples with oxalate averaged 0.061 U higher and at 4 weeks 0.11 U higher than the controls. The initial determination of red cell ATP and 2,3-DPG levels was made after the anticoagulated blood had been held at room temperature for approximately two hours. It was apparent that ATP levels in blood collected into oxalate-containing anticoagulants had already declined substantially in this brief time. This immediate effect of oxalate on red cell ATP levels was confirmed in other studies (data not shown). Over the 4-week storage period oxalate greatly improved preservation of 2,3-DPG but had a markedly deleterious effect on ATP levels. The same experiment was carried out with a final oxalate concentration of only 50 μmol/L. The results of this study are shown in Fig 2.

The effect of oxalate on red cell enzyme activities. The effect of 500 μmol/L potassium oxalate on red cell enzyme activities is summarized in Fig 3. The enzyme most sensitive to inhibition by oxalate was found to be lactate dehydrogenase (LDH), followed closely by monophosphoglyceromutase (MPGM) and then by PK. In contrast to the effect of oxalate, 5 mmol/L malonate failed to inhibit erythrocyte MPGM, PK, or LDH activity. The effect of oxalate on PK kinetics is shown in Fig 4. The inhibition of PK by oxalate was competitive with PEP in the presence of FDP, the allosteric effector of PK, but noncompetitive in the absence of FDP.

The effect of oxalate on red cell metabolism at 37°C. Estimation of the levels of metabolic intermediates other than ATP and 2,3-DPG at the end of the 28-day storage period proved to be unreliable because of the very large amounts of pyruvate and lactate that had accumulated.

Fig 2. The same as Fig 1, except that the oxalate concentration was only 50 μmol/L.

However, studies of the effect of oxalate on red cell intermediates could readily be carried out on red cells incubated in autologous plasma for several hours at 37°C, since a rise in the red cell 2,3-DPG level was demonstrable under these conditions. It was necessary to use an anticoagulant other than CPD for such studies, because of the very substantial effect that temperature exerts on the pH of blood. It is -0.7 pH units higher at 4°C than at 37°C and the citrate preservatives provide an unsuitable medium for study of metabolism at the latter temperature. A suspension of red cells in EDTA anticoagulant plasma incubated under a suitable CO2-air atmosphere is very suitable for such investigations. Preliminary studies showed that maximum elevation of red cell 2,3-DPG levels could be observed after four to six hours incubation at 37°C and that the amount of phosphate in plasma was insufficient to support an optimal effect of oxalate on 2,3-DPG concentration.

Erythrocytes from blood drawn into EDTA anticoagulant were resuspended at a hematocrit of 30% in their own plasma. Sufficient KH2PO4/K2HPO4 pH 7.4 was added to provide a 2.5 mmol/L increase in P04 concentration. Aliquots of the cell suspension were incubated at 37°C in a shaking incubator in an atmosphere of 5% CO2/95% air with
and without the addition of 2 mmol/L oxalate. The possible limiting effect of phosphate and of NAD$^+$ was investigated in incubation mixtures to which excess phosphate or pyruvate had been added. The pH and the levels of metabolic intermediates are presented in Table 1. Figure 5 represents a "cross-over" plot$^{5}$ based on these data. In a similar short-term incubation experiment malonate failed to exert any effect on the level of 2,3-DPG.

The effect of oxalate on red cell survival. Twenty milliliters of rabbit blood was collected into 2.8 mL of CPD solution and divided into bags to which no oxalate was added and those to which sufficient potassium oxalate was added to provide a final concentration of 500 μmol/L. At the end of 21 days storage the survival of the red cells of one of the samples was measured in a normal recipient rabbit, and at the end of 22 days the other sample was labeled with a larger amount of $^{51}$Cr and viability again determined in a rabbit. The order of presentation was varied so that two of the rabbits received red cells that had been stored with oxalate on day 21 and the other two those that had been stored with oxalate on day 22. The results of these studies are presented in Table 2. Shown also is the effect of storage of rabbit blood on red cell ATP and 2,3-DPG levels. Oxalate exerted the same favorable effect on 2,3-DPG concentrations and unfavorable effect on ATP concentration of rabbit erythrocytes as were observed with human cells.

DISCUSSION

Although oxalate has long been used as an anticoagulant for the collection of blood for diagnostic studies, no thought has apparently been given to its use for blood storage. This has probably been due to the presumed toxicity of oxalate. However, the concentration of oxalate required to aid in the maintenance of red cell 2,3-DPG levels is much lower than that required as an anticoagulant. Even a concentration of only 0.5 μmol/L exerted a substantial favorable affect on red cell 2,3-DPG: after 21 days' storage the levels had declined by only about one-third. Assuming uniform distribution throughout body water, the instant administration of 450 mL of blood containing this concentration of oxalate would result in a rise of only ~0.6 micromolar in the oxalate level, an increase of the order of 20%. One must take into account, of course, the effect of the administration of multiple units of blood, the rate of clearance of oxalate in normal and diseased individuals, and the existence in the population of many individuals who may have hereditary abnormalities in oxalate transport$^{6}$ potentially leading to calcium oxalate stone formation.

Table 1. Levels of Metabolic Intermediates in Red Cell Suspensions Incubated With and Without Oxalate and the Indicated Additives

<table>
<thead>
<tr>
<th></th>
<th>2.5 mmol/L Phosphate</th>
<th>10 mmol/L Phosphate</th>
<th>2.5 mmol/L Phosphate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Oxalate</td>
<td>Control</td>
</tr>
<tr>
<td>Glucose-6-phosphate (nmol/mL)</td>
<td>63.78</td>
<td>24.84</td>
<td>65.93</td>
</tr>
<tr>
<td>Fructose-6-phosphate (nmol/mL)</td>
<td>8.68</td>
<td>9.80</td>
<td>29.67</td>
</tr>
<tr>
<td>Fructose-1-6-diphosphate (nmol/mL)</td>
<td>2.55</td>
<td>14.56</td>
<td>11.5</td>
</tr>
<tr>
<td>Dihydroxyacetone-phosphate (mmol/mL)</td>
<td>31.38</td>
<td>779.83</td>
<td>36</td>
</tr>
<tr>
<td>Glyceraldehyde-3-phosphate</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
<tr>
<td>2,3 Diphosphoglycerate (μmol/gHb)</td>
<td>13.82</td>
<td>18.27</td>
<td>11.45</td>
</tr>
<tr>
<td>3-Phosphoglyceric acid (nmol/mL)</td>
<td>98.65</td>
<td>560.84</td>
<td>81.1</td>
</tr>
<tr>
<td>2-Phosphoglyceric acid (nmol/mL)</td>
<td>8.46</td>
<td>36.24</td>
<td>7.99</td>
</tr>
<tr>
<td>Phosphoenolpyruvate (nmol/mL)</td>
<td>15.86</td>
<td>161.66</td>
<td>7.53</td>
</tr>
<tr>
<td>Pyruvate (μmol/L)</td>
<td>328.9</td>
<td>46.50</td>
<td>254.4</td>
</tr>
<tr>
<td>Lactate (μmol/L)</td>
<td>7320.65</td>
<td>8532.15</td>
<td>7460.9</td>
</tr>
<tr>
<td>pH at t = 6 hr</td>
<td>7.506</td>
<td>7.498</td>
<td>7.668</td>
</tr>
</tbody>
</table>
such substances. Dihydroxyacetone is converted to DHAP by red cell triokinase. Inosine undergoes phosphorolysis to ribose-1-phosphate and hypoxanthine, and the ribose-1-phosphate is converted in the hexose monophosphate pathway to F-6-P and to GAP. In each of these cases use of the new substrate is less sensitive to a fall in the pH of stored blood than is glucose, providing a distinct metabolic advantage to the stored erythrocytes. An alternative to providing a more efficient substrate is modifying red cell preservatives so as to inhibit or to stimulate reactions that influence 2,3-DPG synthesis or degradation. In the past, manipulations of hydrogen ion, pyruvate, and phosphate concentrations have been used for this purpose. Increasing the pH stimulates the diphosphoglycerate mutase (DPGM) and inhibits the DPGP reactions. Pyruvate serves to oxidize NADH to NAD, thereby increasing the level of one of the required co-factors for the glyceraldehyde phosphate dehydrogenase (GAPD) reaction. The product of this reaction, 1,3-DPG is the immediate substrate for the formation of 2,3-DPG. Phosphate stimulates glycolysis, probably largely by relieving the inhibition of hexokinase by G-6-P.

It is the DPGM and DPGP reactions that directly control the level of 2,3-DPG in erythrocytes, but these reactions, in turn, are influenced by other metabolic changes in the erythrocyte. PK deficiency is associated classically with increased 2,3-DPG levels, sometimes even in the heterozygous state. The effect of PK deficiency is presumably due to the accumulation of substrates proximal to the PK reaction, i.e., PEP, 2-PGA, and 3-PGA. The latter compound inhibits DPGP and stimulates DPGM. Vora recently suggested that inhibitors of this reaction might be useful in maintaining 2,3-DPG levels during blood storage.

In the present investigation we extended the observations of Kandler et al. Red cell membranes are readily permeable to the oxalate anion, even at 0°C. Oxalate increases 2,3-DPG levels not only in blood stored in an artificial additive solution, but also in CPD anticoagulant. Although ATP depletion accompanied the improved 2,3-DPG maintenance, red cell viability was not impaired, at least in rabbits. Oxalate did not exert its effect by altering intracellular pH. It is a known inhibitor of both plasma LDH and of L type (red cell) PK. We found that not only these enzymes but also red cell monophosphoglycerate mutase is inhibited by oxalate. Our kinetic studies indicated that the inhibition of PK is competitive with respect to PEP when the allosteric effector, FDP is present, a finding that is consistent with data obtained earlier in the study of L type PK.

At 37°C oxalate increases the 2,3-DPG levels of red cells incubated in their own plasma even for a few hours. This provided us with the opportunity of addressing the question of how oxalate accomplishes its effect on 2,3-DPG levels. The studies in whole red cells pin-point the major functional lesion induced by oxalate to the PK reaction: the cross-over plot (Fig 5) shows that the concentration of intermediate compounds proximal to the PK reaction is increased and that those distal to the reaction are decreased. However, a second cross-over was also present at the GAPD step. A deficiency of either phosphate or NAD could produce such an effect. The cross-over before GAPD is not eliminated by increasing

Fig 5. A crossover plot demonstrating the effect of oxalate after six hours incubation in autologous plasma at 37°C under 95% air-5% CO₂. The effect of added P₃ and pyruvate are shown: concentrations represent the amount added to those already present in plasma.

Table 2. The Effect of Oxalate on the Storage of Rabbit Blood

<table>
<thead>
<tr>
<th>Storage Medium</th>
<th>Day 0</th>
<th>Day 21</th>
<th>Viability (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CPD*</td>
<td>7.34</td>
<td>1.76</td>
<td>71.2</td>
</tr>
<tr>
<td>CPD + oxalate</td>
<td>7.34</td>
<td>0.72</td>
<td>72.6</td>
</tr>
</tbody>
</table>

*The same animals served as controls in a concurrent investigation of BW440C.
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the phosphate concentration by 7.5 mmol/L. However, increasing the available NAD by the addition of pyruvate greatly decreased the accumulation of substrates proximal to the GAPD reaction. Thus, it appears that in addition to its effect on the PK reaction, oxalate produces a change in the NADH/NAD ratio of red cells, possibly merely by inhibiting the LDH reaction.

Although the potential toxicity of oxalate might preclude its clinical use, its effect suggests that other PK inhibitors might be useful, if one that was sufficiently nontoxic could be found. It is of interest, in this respect that malonate, the structure of which superficially resembles PEP more closely than does that of oxalate, has no effect on either the PK reaction or on 2,3-DPG levels in incubated red cell suspension. Oxalate is a normal plasma constituent, circulating at a level of ~4 μmol/L. It may be that this compound may exert some in vivo regulatory effect, and further studies are underway to explore this possibility.

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