Control of Red Cell Glycolysis

THE CAUSE OF TRIOSE PHOSPHATE ACCUMULATION*

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SUMMARY

Fructose diphosphate and the triose phosphates are known to accumulate in red blood cells when they metabolize glucose at high rates due to the presence of orthophosphate. This has led to the suggestion that either glyceraldehyde phosphate dehydrogenase or glycerate phosphate kinase is rate-limiting under these conditions. However, the present work shows, by a study of isotope mixing rates and mass action ratios, that under conditions of triose phosphate accumulation both steps proceed much more rapidly than the limiting rate of lactate production. It is observed that the conditions of triose phosphate accumulation also result in net synthesis of glyceraldehyde 2,3-diphosphate and in the occurrence of extremely high ratios of lactate to pyruvate, i.e. DPNH to DPN+, thus explaining how the triose phosphates can be so elevated and yet be at equilibrium. The net synthesis of glyceraldehyde 2,3-diphosphate, which requires an unknown endogenous oxidative system, is presumed to occur because the glyceraldehyde 1,3-diphosphate level exceeds that of the cell in vivo. This results at high glycolytic rates because the glyceraldehyde 1,3-diphosphate steady state concentration is determined by equilibria with the steady state concentrations of phosphoenolpyruvate, ATP, and ADP, which increase, decrease, and increase, respectively, under high glycolytic rate conditions (Rose, I. A., and Warms, J. V. B., J. Biol. Chem., 241, 4848 (1966)). Thus, these effects are not evidence of rate-limiting processes in the utilization of triose phosphates as previously supposed, but rather are consequences of the control of the rates of pyruvate kinase and glyceraldehyde diphosphate mutase by the concentration of their substrates and of the inability of the red cell, in vitro, to maintain the DPNH to DPN+ ratio at its in vivo level in the face of net synthesis of glyceraldehyde 2,3-diphosphate.

Adult human red blood cells, when studied in buffered 0.9% NaCl medium, carry out glycolysis without the accumulation of fructose 1,6-diphosphate, unless the rate of glucose utilization exceeds about 2.5 µmoles per hour (1). When stimulated to this extent with high orthophosphate in the medium, the accumulation of FDP* and triose phosphate proceeds to high concentrations (1, 2) with no parallel increase in the rate of lactate production above a rate of about 4 µmoles per hour. These results are suggestive of a rate-limiting step in the series between glyceraldehyde-3-P and enolpyruvate-2-P, since otherwise mass action would lead to acceleration of the pyruvate kinase step, which is known to operate in first order response to enolpyruvate-2-P in the normal cell (1). Since neither enolpyruvate-2-P nor glyceraldehyde 3-phosphate rises in parallel with the triose phosphates under these conditions, the critical step would seem to be either glyceraldehyde-P dehydrogenase or glycerate-P kinase (1, 3).

Upon the addition of pyruvate to such cells the triose phosphates are observed to fall rapidly to a very low steady state level, suggesting that the availability of DPN+, and hence the combination of lactate dehydrogenase and glyceraldehyde-P dehydrogenase, was limiting the availability of glyceraldehyde 1,3-diphosphate to the kinase step (1). However, Eckel et al. (4), using red cells from defibrinated blood that had been stored in the cold for several days, a procedure that leads to the accumulation of triose phosphates, observed that the rapid loss of triose-P, upon addition of pyruvate, was accompanied by an equivalent loss of pyruvate from the system by its conversion to lactate. This suggested that triose-P contributed the reducing equivalents but not the pyruvate to the lactate dehydrogenase step, since the pyruvate should have been regenerated through glycerate-P kinase and pyruvate kinase to the same extent that pyruvate was reduced. The missing carbon was found in the

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1 The abbreviations used are: FDP, fructose 1,6-diphosphate; PGA, glycerate phosphate; DPG, glyceral diphosphate.
2,3-DPG of the cell. Comparable experiments by Saito and Minakami (3) with cells metabolizing glucose showed that methylene blue stimulated the production of 2,3-DPG but not pyruvate and lactate, and led to a lower level of dihydroxyacetone-P and FDP. Such experiments appear to indicate that the flow of 1,3-DPG through glycerate-P kinase is the step limiting the use of triose-P in the absence of an exogenous oxidant.

In spite of these indications the present study reaches the conclusion that both the dehydrogenase and phosphoglycerate kinase steps are essentially at equilibrium under conditions of triose-P accumulation and certainly would be so in the circulating cell under normal conditions of glycolysis. The failure of added oxidant to stimulate flow through the regular glycolytic path is here attributed to rate limitation at the phosphoglycerate kinase step under these conditions only.

METHODS

Determination of Glycerate-1,3-dicarboxylic Acid in Cell Extracts—1,3-DPG was found to be optimally stable at pH 7.5 to 8.5, with a half-life for hydrolysis of 40 min at 37° and 3 hours at 25°. Rapid hydrolysis did not occur within the range pH 2 to 10.5 when the temperature was kept low, in confirmation of the report of Negelein and Brömel (5). A satisfactory procedure for the preparation of cell extracts and for the assay of 1,3-DPG was the following. Four volumes of 25 to 33% cell suspension were added to 1 volume of cold 50% trichloroacetic acid containing 0.01 mM EDTA, and the mixture was cooled rapidly at -10° and then spun in a refrigerated centrifuge. The supernatant was removed and extracted three times with an equal volume of ether to remove the acid. It was then neutralized by adding triethanolamine-HCl, pH 8.0, to a final concentration of about 50 mM and enough triethanolamine to bring the pH to about 7.5. The neutralized extract was placed momentarily on a rotating evaporator to remove the ether under slight vacuum. It was next treated for removal of dihydroxyacetone-P and glyceraldehyde-3-P by neutral extract was placed momentarily on a rotating evaporator. For the 1,3-DPG extraction, 1 unit of glycerate-P dehydrogenase (1.5 units per ml), triose-P isomerase (2.4 units), and DPNH, which was added in small amounts until no further reduction was observed in the spectrophotometer. At this point it was usually desirable to adsorb most of the fluorescing materials with acid-washed charcoal (10 mg per ml). The treated supernatant was transferred to a cuvette and DPNH (5 to 10 mmoles) was added. A rapid decrease in fluorescence is observed upon the addition of glyceraldehyde-P dehydrogenase (0.3 unit per ml). A known amount of 1,3-DPG (1 to 2 mmoles) was then added to the cuvette as a standard for comparison. Every effort was made to work quickly and maintain the extract at ice temperature prior to the point of enzymatic reaction. 1,3-DPG-14C, prepared and maintained according to the method of Rose (6), was added to the cold trichloroacetic acid cell suspension and carried through to the neutralized extract. It was then treated with ADP plus glycerate-P kinase, and radioactivity removed by charcoal was taken as a measure of ATP. When compared with the untreated stock solution, about 80 to 85% of the acyl-

Evidence Suggestive of Rate-limiting Glycerate-P Kinase Step—It can be demonstrated, using glucose as the carbon source, that the net flow through the glycerate-P kinase step is not increased by the addition of pyruvate, although the rate of lactate production is increased. Thus, in Table I, the increased lactate production is accounted for by pyruvate consumed, which precisely equals the increased amount of 2,3-DPG. The glycolate-6-P shunt pathway accounts for only 4% of the glucose utilization even in the presence of pyruvate and cannot account for much of the pyruvate consumed. The less-than-expected amount of lactate in the nonpyruvate case is the result of FDP and triose-P accumulation in such cells (1, 2), and the higher rate of glucose utilization in these cells is attributed to the stimulation of fructose-6-P kinase by the FDP (1). The flow through the glycerate-P kinase and pyruvate kinase steps is the same in the two cases (Table I, B minus C). The equal rates for pyruvate kinase (0.068 pmole per min per ml of cells) appear to be the consequence of compensating changes of ADP and of enolpyruvate-2-P (the latter change being more precisely shown in the amount of 3-PGA). Whole cell studies have shown that the enolpyruvate 2-P concentration of the normal cell is a first order determinant of the pyruvate kinase rate (1). In addition, it is probable from kinetic studies with the isolated enzyme and from estimates of the concentration of the Mg++ in the cell, that ADP Mg++ is also in the range of concentration that controls the rate of this step.

Two approaches are available for the examination of the rates of component reactions of a system in the steady state. One is the study of the rate of flow of isotope from one cell pool to the other. The second makes use of the concentrations of intermediates to compare the mass action ratio with the known equilibrium constant for each step.

Isotope Exchange Studies—The flow of isotope between intracellular P and ATP would serve as a ready measure of the dehydrogenase-kinase couple. When 14C-P is added to cells in the state of triose-P accumulation resulting from incubation with high levels of P, the rate of labeling of ATP based on the specific activity of the intracellular P is considerably greater than expected from the net glycolytic rate of the cell (Table II) and is undoubtedly attributable to the exchange process dependent on the dehydrogenase and kinase (9). As expected, the apparent exchange rate is observed to fall as the two pools (P and ATP) approach each other in specific activity. Thus, a value closer to 2.3 pmole per min per ml of cells should represent the true rate of exchange. The calculation of rate depends on the assumption that the fully mixed cellular P is the source of the P of the dehydrogenase step, an assumption that has been frequently eschewed by earlier authors, based on the presumed occurrence
Red cells in a 30% suspension in 50 mm Pi medium, pH 7.8, with 2.5 mm glucose or glucose-1-14C (in one experiment) and with 1.5 mm sodium pyruvate or in its absence (control) were flushed with argon for 10 min at 0° and incubated for 140 min at 37°. Samples were taken at 0 min and 140 min to determine pyruvate in the medium from which the cells were removed by centrifugation, and other samples were put into HClO4 or trichloracetic acid for the preparation of acid extracts used in the other analyses. An alkaline center well was used in the 14C-glucose experiment to absorb the 14CO2 generated by acid addition to this incubation at 140 min.

**TABLE I**

<table>
<thead>
<tr>
<th>Component measured</th>
<th>Control</th>
<th>4-Pyruvate</th>
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<tbody>
<tr>
<td>Glucose consumed</td>
<td>6.65</td>
<td>5.54</td>
</tr>
<tr>
<td>Lactate formed</td>
<td>9.70</td>
<td>11.70</td>
</tr>
<tr>
<td>Pyruvate consumed</td>
<td>2.14</td>
<td></td>
</tr>
<tr>
<td>Flow through pyruvate kinase, B - C.</td>
<td>9.70</td>
<td>9.80</td>
</tr>
<tr>
<td>2,3-DPG at 140 min</td>
<td>5.46</td>
<td>5.91</td>
</tr>
<tr>
<td>2,3-DPG at 0 min</td>
<td>3.71</td>
<td>3.75</td>
</tr>
<tr>
<td>2,3-DPG at 30 min</td>
<td>0.25</td>
<td>2.16</td>
</tr>
<tr>
<td>2,3-DPG at 157 min</td>
<td>0.076</td>
<td>0.043</td>
</tr>
<tr>
<td>ATP</td>
<td>1.23</td>
<td>1.10</td>
</tr>
<tr>
<td>3-PGA</td>
<td>0.068</td>
<td>0.094</td>
</tr>
</tbody>
</table>

**TABLE II**

Rate of 32P-P-ATP exchange

Washed cells were incubated for 2 hours at 37° in glucose with high Pi medium, pH 7.8, resulting in cells of high content of Pi, 10.6 μmoles per ml of cells and of high triose-P. The cells were washed with 0.9% NaCl (once at room temperature) and with mannitol-imidazole-glutamate medium, pH 7.03 (0.17 M, 0.05 M, and 0.02 M, respectively) twice at room temperature. The cells were added to an equal volume of this medium containing 32P (1 mm, 3.2 X 106 cpm per μmole) at 38°. Samples of 1 ml were placed at 0 min, 15 sec, 30 sec, and 1 min into either 1 ml of 20% trichloroacetic acid for the determination of acid-labile organically bound 32P (i.e., not extractable by the Berenblum and Chain extraction of Pi) or into 10 ml of cold 0.9% NaCl solution. The radioactive cells and the unlabeled cells were subsequently introduced into the medium recovered from the other cells and glucose added to start each subsequent incubation of "hot" cells in "cold" medium and "cold" cells in "hot" medium. The incorporation of radioactivity in the two "identical" incubations generates two simultaneous equations that relate the total counts incorporated into ester phosphate form to the determined average specific activities of Pi of the medium and cells during the interval sampled and the unknown rates of incorporation from the cells (C) and the medium (M); Thus, in the 0- to 5-min interval

\[
\begin{align*}
7,290 - 3,980 & = 7,000 \cdot C \cdot 5 + \frac{157 - 19}{2} \cdot M \cdot 5 \\
8,570 - & \frac{13,600}{2} - C \cdot 5 + 623,000 \cdot M \cdot 5
\end{align*}
\]

Solving the simultaneous equations for C and M gives C = 0.09 μmole per min per ml of cells and M = 0.001 μmole per min per ml of cells. Thus, the esterification of Pi is 99% by way of the completely mixed intracellular pool of Pi. The prolonged incorporation of 32Pi observed in the hot cell experiment is due in large part to the increase in total ATP in these depleted cells so that one is observing net synthesis coupled to glycolysis rather than the exchange that was seen in Table II.

**TABLE III**

Utilization of Pi of medium for glyceraldehyde-P dehydrogenase

Washed red cells were incubated at 37° for 90 hours in 20 mm Pi medium, pH 7.4, in the absence of glucose. This serves to lower the ATP and dissipate any glycolytic reserves that contribute to rapid "exchange" esterification of Pi. Half the cells were then suspended in pH 7.4, 20 mm Pi, 0.21 M sucrose medium and half in the similar but unlabeled medium. After 5 min at 37° the supernatant was removed and the cells were washed with cold NaCl solution. The radioactive cells and the unlabeled cells were subsequently introduced into the medium recovered from the other cells and glucose added to start each subsequent incubation of "hot" cells in "cold" medium and "cold" cells in "hot" medium.

The incorporation of radioactivity in the two "identical" incubations generates two simultaneous equations that relate the total counts incorporated into ester phosphate form to the determined average specific activities of Pi of the medium and cells during the interval sampled and the unknown rates of incorporation from the cells (C) and the medium (M); Thus, in the 0- to 5-min interval

- A 0.25 min, (3,100)/(10,800) = 0.57 μmole/0.25 min = 2.3 μmoles per min.

of glycolysis at the cell membrane (11-13). In behalf of this assumption it should be said that in many experiments in this laboratory, using cells with Pi elevated initially to manageable levels, it has never been noted that the ATP-Pi position exceeds the levels, it has never been noted that the ATP-Pi position exceeds
The same under anaerobic conditions or in the presence of 0.1 mM ouabain, the rate of the aldolase and isomerase steps may be conveniently analyzed by determining the rate of tritium exchange between tritiated water and FDP. Tritium could be introduced into the C-3 and C-5 positions of FDP by action of isomerase acting on dihydroxyacetone-P and converting it to glyceraldehyde-3-P. Cells were incubated to accumulate triose phosphates and FDP at high Pi, then washed and transferred to a high P1 medium, pH 7.4, containing glucose and 2H2O. After 1 min the cells were acidified and FDP + 2,3-DPG was isolated from the acid extract. The rate of labeling of 2,3-DPG was calculated from the total counts in the 2,3-DPG and the time average specific activity of the 3-carbon unit of FDP, giving an approximate rate which requires correction for the loss of counts from the DPG during the steady state metabolism of DPG with its time average specific activity.

<table>
<thead>
<tr>
<th>Time</th>
<th>Specific activities</th>
<th>Pool size</th>
<th>Mixing ratea</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>cpm/mole of 3 carbons</td>
<td>μmoles/mi cells</td>
<td>μmoles/min/mi cells</td>
</tr>
<tr>
<td>min</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>77,500</td>
<td>18,700</td>
<td>2.0</td>
</tr>
<tr>
<td>10</td>
<td>112,000</td>
<td>46,600</td>
<td>2.1</td>
</tr>
</tbody>
</table>

* Rate = (counts in DPG)/[triose-P]i time

per min per ml of cells is obtained. This rate cannot be attributed to any significant extent to the net synthesis of 2,3-DPG since, to this extent, a net fall in total triose-P would have been noted and was not. Thus, the rates of glucose utilization and lactate production differ only very slightly compared to this exchange rate. In a further study of the same kind, with cells at pH 7.4, an exchange rate of 0.28 to 0.30 was obtained that was the same under anaerobic conditions or in the presence of 0.1 mM ouabain.

The rate of the aldolase and isomerase steps may be conveniently analyzed by determining the rate of tritium exchange between tritiated water and FDP. Tritium could be introduced into the C-3 and C-5 positions of FDP by action of isomerase at the triose-P stage before conversion to lactate through the action of glyceraldehyde-P dehydrogenase and lactate dehydrogenase.

The FDP + 2,3-DPG exchange is a function of the rates of five steps: aldolase, triose-P isomerase, glyceraldehyde-P dehydrogenase, glyceraldehyde-P kinase, and glyceral-P mutase. The rate of the aldolase and isomerase steps may be conveniently analyzed by determining the rate of tritium exchange between tritiated water and FDP. Tritium could be introduced into the C-3 and C-5 positions of FDP by action of isomerase at the triose-P stage and into C-4 by action of aldolase and triose-P isomerase acting on dihydroxyacetone-P and converting it to glyceraldehyde-3-P. Cells were incubated to accumulate triose phosphates and FDP at high P1, then washed and transferred to a high P1 medium, pH 7.4, containing glucose and 2H2O. After 1 min the cells were acidified and FDP (0.78 μmoles per ml of cells) was isolated. It had a specific activity of 31,600 cpm per μpmole. When the FDP was treated with aldolase and α-glycerol-P dehydrogenase, no counts were made volatile until the further addition of triose-P isomerase, which resulted in the labilization of 56% of the counts from the original C-5 position of FDP, which would then have 17,000 cpm per μpmole at that position. One may calculate the exchange rate between C-5 of FDP and 2H2O to be:

\[ r = \frac{-2.3 \cdot [A] [B]}{[A] + [B]} \cdot \log (1 - f) \]

Thus, the isomerase rate is more than twice as great since this calculation does not include radioactivity in C1-3 half of FDP (at C-3), the radioactivity in free dihydroxyacetone-P, nor that represented in metabolites of glyceraldehyde-3-P. Thus, the aldolase-isomerase steps, even without correction for a 7-fold isotope discrimination against 2H in isomerase reaction (15), are many times greater than the rate of FDP to lactate, about 0.07 μmole per min. A similar conclusion was reached in a previous study (10) in which the 4H-glucose that was metabolized was observed to lose almost all of its radioactivity to water due to the combined action of triose-P isomerase and aldolase at the triose-P stage before conversion to lactate through the action of glyceraldehyde-P dehydrogenase and lactate dehydrogenase.

In this same experiment, the 2,3-DPG (4.55 μmolecules per ml of cells) had a specific activity of 4,850 cpm per μpmole after 1 min of incubation in 2H2O. This corresponds to a corrected exchange rate of 0.82 μmole per min per ml of cells.

Thus, the glyceral-P mutase step must be mixing radioactivity between the monophosphoglycerates and 2,3-DPG at greater than 0.8 μmole per min per ml of cells, since the measured rate includes additional steps. Previous studies (1, 17) have established, from the mass action ratio of the enolase and glyceral-P mutase reactions, that these steps are approximately at equilibrium. The rapid exchange of tritium between water and 2,3-PGA provides independent support for this conclusion.

From these minimum rates one may calculate the maximum rate necessary for the combined glyceraldehyde-P dehydrogenase and glyceral-P kinase steps as follows: assuming quasi-equilibrium, the isotope exchange rate of steps in series add reciprocally (18) as follows:

\[ \frac{1}{V_{\text{FDP}}} + \frac{1}{V_{\text{GPH}}} + \frac{1}{V_{\text{GPH}-\text{3PGA}}} = \frac{1}{V_{\text{FDP}} + \text{GPH} + \text{GPH}-\text{3PGA} + \text{3PGA}-\text{1,3-DPG}} \]

\[ \frac{1}{V_{\text{FDP}}} + \frac{1}{V_{\text{GPH}}} + \frac{1}{V_{\text{GPH}-\text{3PGA}}} < \text{1.1 μmole per min per ml of cells} \]

Thus, the rate of glyceraldehyde-3-P → 3-PGA under conditions of FDP accumulation falls in the range 0.29 to 1.1 μmolecules per min per ml of cells, which is 4 to 15 times greater than the rate of lactate production. It would be inferred from these considerations that the previously held conclusion of a rate-limiting step in the dehydrogenase-kinase couple, proceeding at the limiting rate of lactate production, 0.07 μmole per min per ml of cells, cannot be valid.

**Mass Action Ratio of Glyceraldehyde-P Dehydrogenase Reaction**—The argument previously presented to explain the accumulation of triose phosphates was that at the higher glycolytic rates, due to increased P1, the cells accumulated more oxidized intermediates such as P-glycerate and enolpyruvate-2-P to support the increased rate of pyruvate kinase and, that due to the lack of an oxidizing system in red cells, this forced the ratio of DPNH to DPN+ to increase, resulting in a slowing in glyceraldehyde-P dehydrogenase. The DPNH to DPN+ ratio can be

\[ \frac{-2.3 \cdot 0.78 \cdot \log (1 - \frac{17,000}{29,000})}{1} = 0.74 \text{ μmole/min/ml cells} \]
Estimated from the lactate to pyruvate ratio since the lactate dehydrogenase step is very rapidly catalyzed compared with the rate of pyruvate formation in these cells and since there are no competing reactions for lactate and pyruvate, there is adequate time for adjustment of the lactate dehydrogenase equilibrium.

The data are presented in this way since it is not possible to obtain measurements of DPIN and DPNH that represent kinetically effective concentrations (19, 20). It is observed that the DPNH to DPN+ ratio as represented by the lactate to pyruvate ratio does indeed rise greatly in the high Pi cells, and in fact tends to parallel the rise in triose-P (Table V), suggesting that the two factors are related. However, the inference that this correlation is the result of an inhibition of glyceraldehyde-P dehydrogenase by DPNH is not correct, as seen in Table VI. A determination of the components of the glyceraldehyde-P and lactate dehydrogenase reactions was made to eliminate the DPNH to DPN+ ratio and II+ concentration between them and allow the calculation of a combined mass action ratio. The published equilibrium values for the two dehydrogenases provide a range of values surprisingly near the determined quotient of the two mass action ratios. Thus, the only reported value estimated red cells in the absence of added oxidant has been noted by I. A. Rose and J. V. B. Warms.

### Table V

**Effect of Pi on lactate to pyruvate ratio of glycolyzing cells**

Cells were incubated in 10 mm Pi medium, pH 7.4, for 1 hour at 37°C with glucose and then diluted to a 20% hematocrit in the same medium or one containing 50 mm Pi. Glucose was 1 mm. Samples were then taken for determination of lactate and pyruvate of the medium, of Pi of cells washed with cold 0.9% NaCl solution, and of acid-soluble intermediates. ATP and ADP at a ratio of about 15 did not differ significantly in the two incubations. The rate of lactate production was 1.6-fold greater in the high Pi cells (0.045 and 0.072 amole per min per ml of cells) which could be attributed to the 1.6-fold higher enolpyruvate-2-P level (18 and 30 amoles per ml of cells).

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Medium — 10 mm Pi</th>
<th>Medium — 50 mm Pi</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>F&lt;sub&gt;i&lt;/sub&gt;</td>
<td>Total triose-P</td>
</tr>
<tr>
<td>1</td>
<td>3,060</td>
<td>117</td>
</tr>
<tr>
<td>10</td>
<td>3,730</td>
<td>6</td>
</tr>
<tr>
<td>20</td>
<td>3,730</td>
<td>6</td>
</tr>
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</table>

### Table VI

**Mass action ratio of glyceraldehyde-P dehydrogenase in high Pi cells**

The high Pi medium conditions of Table V were repeated in this experiment.

<table>
<thead>
<tr>
<th>Time (hr)</th>
<th>F&lt;sub&gt;i&lt;/sub&gt;</th>
<th>Lactate</th>
<th>Pyruvate</th>
<th>Glyceraldehyde-3-P</th>
<th>1,3-DPG</th>
<th>Mass action ratio: (K&lt;sub&gt;GPDH&lt;/sub&gt;:K&lt;sub&gt;LDH&lt;/sub&gt;)&lt;sup&gt;e&lt;/sup&gt;</th>
<th>Total triose-P</th>
</tr>
</thead>
<tbody>
<tr>
<td>1/2</td>
<td>6,200</td>
<td>2,850</td>
<td>1.65</td>
<td>31</td>
<td>1.1</td>
<td>6.5</td>
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<tr>
<td>1</td>
<td>10,900</td>
<td>6,650</td>
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<td>63.7</td>
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<tr>
<td>2</td>
<td>11,500</td>
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<td>13,300</td>
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<td>61</td>
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<td>4.5</td>
<td>2.56</td>
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<tr>
<td>4</td>
<td>11,600</td>
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<td>5</td>
<td>85.5</td>
<td>2.7</td>
<td>6.7</td>
<td>2.51</td>
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</table>

*Mass action ratio: (K<sub>GPDH</sub>:K<sub>LDH</sub>)<sup>e</sup> = (lactate-1,3-DPG)/(1.5-pyruvate-P; glyceraldehyde-3-P); the value of 1.5 is used to convert from amount per ml of cells to concentration. GPDH, glyceraldehyde-P dehydrogenase; LDH, lactate dehydrogenase.*

### Notes

1. It is evident that the red cell, lacking an aerobic oxidative system, must have another means for reoxidation of the DPNH required for this further accumulation to occur. A similar imbalance in oxidized products of glycolysis was previously reported, especially with rapidly glycolyzing cells and cells with abnormally low pyruvate kinase levels (1). It was not possible to attribute the DPNH oxygen to glycerol-P dehydrogenase, nor does the absence of oxygen diminish the net synthesis of 2,3-DPG. The route of DPNH oxidation in this situation in vitro remains to be determined.

An explanation for the net formation of 2,3-DPG derives from a detailed study of the human red cell glycerate-diphosphate (6). This enzyme is inhibited competitively with respect to 1,3-DPG by concentrations of 2,3-DPG in the physiological range. Thus, there would be increased synthesis of 2,3-DPG at 1,3-DPG concentrations that exceeded the physiological norm responsible for the average steady state level of 2,3-DPG of about 4 umoles per ml of cells. The origin of an increased 1,3-DPG level must now be sought in the condition of increased glycolytic rate.

2. F. A. Oski, unpublished observations.
20% trichloracetic acid and treated further as for the determination of 1,3-DPG was followed. The values for 1,3-DPG are not contained in the high Pi medium with glucose after having accumulated in the presence of added methylene blue or pyruvate at times when the triose-P pool is being rapidly depleted. To analyze the in vivo mass action ratio of red cells, blood freshly drawn into heparin from a brachial vein of a resting subject was rapidly transferred to an equal volume of blood freshly drawn into heparin from a brachial vein of a

<table>
<thead>
<tr>
<th>Oxidant</th>
<th>Sample time</th>
<th>ATP</th>
<th>ADP</th>
<th>1,3-DPG</th>
<th>3-PGA</th>
<th>(1,3-DPG)</th>
<th>(ATP)/(1,3-DPG)</th>
<th>(ADP)/(1,3-DPG)</th>
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<tbody>
<tr>
<td>None</td>
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<td>1340</td>
<td>51</td>
<td>2.6</td>
<td>65</td>
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<td></td>
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<tr>
<td>Methylene blue</td>
<td>1</td>
<td>1340</td>
<td>39</td>
<td>17.0</td>
<td>150</td>
<td>293</td>
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<td></td>
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<tr>
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<td>110.0</td>
<td>86</td>
<td>69</td>
<td></td>
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<tr>
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<td>112</td>
<td>0.52</td>
<td>29</td>
<td>563</td>
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</table>

a Corrected for loss during the extraction.

b Corrected for the breakdown of 1,3-DPG at the time of assay.

**Table VIII: Influence of Mg**

All incubations contained KCl (0.1 M), cysteine (5 mM), rabbit muscle glyceraldehyde-3-P kinase (1 to 3 units per ml, Boehringer Mannheim), and varying total magnesium, [Mg]. The incubations at pH 8.0 contained triethanolamine-HCl (30 mM), ATP (1050 μM), and 3-PGA (3500 μM). Those at pH 7.0 contained imidazole-HCl (30 mM), ATP (1000 μM), and 3-PGA (3100 μM). After 5 min at 37°, trichloracetic acid was added and the procedure for determination of 1,3-DPG was followed. The values for 1,3-DPG are corrected for loss during manipulation. Repeats with five times more enzyme did not contain more ADP or 1,3-DPG.

<table>
<thead>
<tr>
<th>pH</th>
<th>[Mg]</th>
<th>(ATP)</th>
<th>(3-PGA)</th>
<th>1,3-DPG</th>
<th>ADP</th>
<th>K_{eq}</th>
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<tr>
<td></td>
<td>mM</td>
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<td>170</td>
<td>38.0</td>
<td>52.5</td>
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</tbody>
</table>

a Calculated from the dissociation constants: K_{MgATP} = 19 μM, K_{MgATP} = 1.35 mM, and K_{MgATP} = 10^{-1} μM at 37°, 0.1 ionic strength (28).

b K_{eq} = (ATP-3-PGA)/(ADP-1,3-DPG).

products of glyceraldehyde-P kinase were determined in cells maintained in the high Pi medium with glucose after having accumulated high triose-P, and in the presence of added methylene blue or pyruvate at times when the triose-P pool is being rapidly depleted by conversion to 2,3-DPG. The values obtained indicate a definite decrease in the mass action ratio in the presence of oxidant (Table VII). All values for this ratio are below the equilibrium constant of the kinase of 3000 reported by Büchler (26). Since this value was obtained at high (Mg²⁺), and since previous experiments from this laboratory (27) have established that (Mg²⁺) is present at low concentration in the intact cell, probably <50 μM, under conditions of high ATP to ADP ratio, the effect of (Mg²⁺) on K_{eq} was determined (Table VIII). The mass action ratio of 650 obtained in the absence of oxidant and 560 for the whole blood are within a factor of 2 of the low (Mg²⁺) limit for K_{eq} of about 1200. This result would indicate that the forward rate of the kinase is only twice the rate of net flux 4 rather than the 4- to 15-fold range indicated by the isotope mixing experiments. The reliability of the mass action ratio of four components assuming a single pool for each component is probably not sufficient to regard this discrepancy with concern. However, the inability of glyceraldehyde-P kinase to maintain its rate following the large increase in 1,3-DPG after oxidant addition is evident from the considerable fall in the mass action ratio of the kinase of these cells. This explains the failure to achieve increased rates through the pyruvate kinase step (Table I). The major factor limiting glyceraldehyde-P kinase is undoubtedly the low amount of ADP, only part of which is present as ADP-Mg (27). Evidently, at the very unfavorable ATP to ADP ratio >30 the glyceraldehyde-P kinase becomes progressively rate-determining.

**DISCUSSION**

The present study, and indeed all in vitro studies of red cells place the cell in an artificial environment with respect to the availability of oxidant for DPNH of the cell. For the isolated cell, the primary routes for DPNH oxidation are by lactate dehydrogenase action with glycolytically generated pyruvate and thus derived from net breakdown of 2,3-DPG. However, the cell in the circulation has, in addition, the plasma pyruvate which is buffered at a lactate to pyruvate ratio of 10 to 20:1 (8) by the visceral tissues through aerobic metabolism. Thus, a period of net 2,3-DPG synthesis in the circulating cell would not produce the marked change in triose-P seen in vitro (Table VI).

The present study (Table VI), as did the previous one (1), calls attention to an additional means of oxidation of DPNH indicated by the net synthesis of 1 to 2 μmoles of 2,3-DPG from glucose or inosine in the absence of added oxidants. Inadequate amounts of glyceraldehyde-P or glyceraldehyde-P are observed. The nature of the oxidant is not clear, nor is it obvious what physiological role it might play, since this path appears to depend on much higher DPNH to DPN⁺ ratios (~10⁵) than would occur in equilibrium with the plasma oxidation-reduction buffer, ~10⁴ (20).

The data, indicating that the glyceraldehyde-P dehydrogenase and glyceraldehyde-P kinase are at quasi-equilibrium in the in vitro situation that leads to triose-P accumulation and net 2,3-DPG synthesis are of two kinds, isotope exchange and mass action ratios. It is very likely that this is also the state in the cell in vivo. Both glyceraldehyde-P and 1,3-DPG in vivo are below the level of 1 μmole per ml of cells, making their accurate determination in the steady state impossible.

4 As pointed out by Hess and Brand (29), for a sequence in the}

\[
\begin{align*}
\text{R} & \ \overset{1}{\longrightarrow} \ \text{C} \ \overset{n}{\longrightarrow} \ \text{product} \\
-1 & \quad (C/B)_{a} \quad K_{a} = 1 - v_{a}/\psi_{a}
\end{align*}
\]
termination difficult and also casting doubt on rate limitation in either step. The kinase, despite its extremely high maximum activity in the cell, 20 to 30 units per ml of cells (30), does become limiting under conditions of ATP to ADP above 30 (Table VII). The ratio found in freshly drawn cells is usually in the range 5 to 10, and that of cells that are accumulating triose-P in the presence of glucose is 13 to 16 (Table I and Reference 1).

Incubation of such cells in the absence of glucose (Table VII, in the presence of glucose is 13 to 16 (Table I and Reference 1). The ratio found in freshly drawn cells is usually in the limiting under conditions of ATP to ADP above 30 (Table VII). Thus, it seems evident that under physiological conditions the ADP level of the cell is not a kinetic signal or rate-limiting factor for the glycerate-P kinase step in the same sense that it is thought to control phosphofructokinase or even pyruvate kinase.

The role of ADP as a controlling factor in lactate formation seems to be the following. Since pyruvate kinase is operating in the normal erythrocyte below saturation for both ADP and enolpyruvate 2-P, a low cellular ADP is easily compensated for by an increased enolpyruvate-2-P to maintain a steady rate of lactate production. The effect of a low ADP, however, is very sensitively reflected in the 1,3-DPG level, which is related through the glycerate-P kinase equilibrium as 1,3-DPG = (PGA·ATP)/(ADP·Kc). Since 3-PGA follows enolpyruvate-2-P and since enolpyruvate-2-P is inversely related to ADP as indicated above, the 1,3-DPG level, and hence the rate of 2,3-DPG synthesis becomes "doubly" sensitive to changes in ADP concentration as it is determined by the balance of ATP generation and utilization. The level of 1,3-DPG will, of course, also be determined by the cell's content of pyruvate kinase since a low amount of this enzyme will force the enolpyruvate-2-P and hence 3-PGA and 1,3-DPG to high levels and hence this would explain the higher amounts of 2,3-DPG in pyruvate kinase-deficient cells (31).

Since the steps from FDP to enolpyruvate-2-P are therefore concluded to be normally at quasi-equilibrium, which rests on the base of enolpyruvate-2-P and ADP, it is evident that a 1,3-DPG level will not be determined by such factors as Pi, DPN+, or FDP, except insofar as these factors determine the glycolytic rate and affect the ATP, ADP, and enolpyruvate-2-P concentrations.

The present study is one of the few cases where the rates of triose-P dehydrogenase and glycerate-P kinase in the whole cell have been analyzed. Discussion of the regulation of these steps in ascites cells, heart, and brain (7, 32, 33) have not led to a definitive clarification of the state of these reactions largely because of the uncertainty in the levels of 1,3-DPG, DPNI, DPNH, and glyceraldehyde-3-P, a problem that is treated specifically here. In addition, the occurrence in red cells of a large pool of 2,3-DPG in rapid equilibrium with 2- and 3-PGA has permitted the measurement of isotope exchange between FDP and PGA, which gives rise to the major evidence of a rapid rate of these steps relative to the glycolytic rate of these cells.

The present paper demonstrates for the first time that ATP synthesized in glycolysis in the glyceraldehyde-3-P dehydrogenase-phosphoglycerate kinase sequence obtains most, if not all, of its Pi from the mixed pool of the cell rather than from a pool which is in ready equilibrium with medium Pi (Table III). Earlier it had been established by Zipursky and Israels (34), and recently confirmed by Schrier (35), that entry of Pi into the red cell is independent of glycolytic function.

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Control of Red Cell Glycolysis: THE CAUSE OF TRIOSE PHOSPHATE ACCUMULATION
Irwin A. Rose and Jessie V. B. Warms


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