Inorganic Phosphate and Enhanced Glucose Degradation by the Intact Erythrocyte*

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Factors affecting the rate of glucose utilization by the intact mammalian erythrocyte have been the subject of several recent reports (1-3). The role of inorganic phosphate in the stimulation of erythrocyte glucose utilization (4) was examined by Rose, Warm, and O'Connell (2). The ability of Pi to moderate the effectiveness of glucose 6-phosphate as an inhibitor of hexokinase was demonstrated by these workers.

We have examined further the nature of the Pi-induced stimulation of glucose utilization by the intact erythrocyte. It was previously noted (3) that enhanced glucose utilization induced by Pi was accompanied by a characteristic deficiency of lactate production by the cells. The results of further examination of this effect of Pi on erythrocyte glycolysis will be summarized in the present report. Evidence will be presented implicating the enzyme, phosphofructokinase, as a primary site of Pi activation (5, 6), leading to enhanced glucose utilization by the intact erythrocyte.

EXPERIMENTAL PROCEDURE

Erythrocytes—Fresh human blood drawn in ethylenediaminetetraacetate (EDTA, disodium, 1 mg per ml) was used in these experiments. Erythrocytes were separated by centrifugation, washed three times with 10 volumes of 0.9% NaCl solution and the leukocyte-rich top layers were discarded. The cells were used directly after washing.

Incubation—Cells were incubated as 10% suspensions in potassium-free isotonic media with excess glucose (3), glycylglycine buffer at 0.05 M and designated pH, sodium chloride (addition as required), and phosphate where indicated. Incubations were performed for specified periods at 37°. Aliquots of the incubation mixtures were removed for hemoglobin analysis as a more precise measure of the actual erythrocyte concentrations. A 1-ml portion of packed erythrocytes contains approximately 0.35 g of hemoglobin. Thus, for purposes of data evaluation, concentrations expressed per g of hemoglobin represent about three times the amount in each milliliter of packed cells.

Deproteinization, Extraction, and Separation of Metabolites—Following specified periods of incubation, aliquots of the incubation mixture were removed and deproteinized directly with Somogyi reagent (7) for glucose analyses and with trichloroacetic acid for all other analyses. Extraction of intracellular inorganic phosphate and soluble organic phosphates was carried out on cells washed three times with 10 volumes of 0.9% NaCl solution (washings and extraction at near 0°). The washed cells were extracted with 9 volumes of 5% trichloroacetic acid. 2,3-Diphosphoglycerate was separated from trichloroacetic acid extracts as the insoluble barium salt prior to analysis. Analyses of other glycolytic intermediates were carried out on trichloroacetic acid extracts after removal of the acid by repeated ether extractions.

Analytical Methods—Glucose was determined (8) with glucose oxidase (Worthington). Lactate was determined (9) with lactate dehydrogenase (Boehringer und Soehne). Pi was determined by a slight modification (10) of the method of Lowry and Lopez. Organic phosphate was measured as the difference between total phosphate (11) and Pi present in the washed cell extracts. Hemoglobin was measured as cytochromemoglobin (12). Assay of 2,3-diphosphoglycerate was carried out by a colorimetric analysis (13) of the isolated barium salt.

Analyses of all other glycolytic intermediates were carried out by fluorometric measure of DPNH or TPNH appearance or DPNH disappearance upon addition of appropriate enzymes (see References 10 and 14). Fructose 1,6-diphosphate, glyceraldehyde-3-P, and dihydroxyacetone-P were determined by the fluorometric measure of DPNH formation with glyceraldehyde-P dehydrogenase (15) plus appropriate auxiliary enzymes. Glucose-6-P, fructose-6-P, α-glycerol-P and pyruvate were determined according to the methods described by Lowry et al. (10). Glucose 1,6-diphosphate was assayed as glucose-6-P following hydrolysis (15 min at 100° in 0.1 N HCl). All fluorometric measurements were made at 25° with the use of a model 111 Turner fluorometer equipped with a constant temperature door and recorder. Enzymes were products of Boehringer und Soehne or Sigma Chemical Company unless otherwise stated.

Preparation of Phosphofructokinase—Erythrocyte P-fructokinase was prepared in partially purified form by DEAE-cellulose column chromatography as employed previously (16). Enzyme elution from the cellulose column was carried out with 0.1 M K₂HPO₄. Merecaptoethanol was added to the active eluates which were then combined and fractionated with (NH₄)₂SO₄. The fraction obtained between 30 and 45% of saturation was retained and was dialyzed overnight against 0.01 M mercaptoethanol plus 0.01 M Tris buffer at pH 8.0. The dialyzed preparation was essentially free of hemoglobin and contained little detectable aldolase, phosphoglucose isomerase, myokinase, or ATPase activity when tested under the conditions of the P-fructokinase assay. The preparation was considered to be of
sufficient purity to allow reliable measurements of P-fructokinase action and was used directly. The enzyme preparation synthesized approximately 20 μmoles of fructose 1,6-diphosphate per min per mg of protein when assayed in the presence of 1 mM ATP, 5 mM fructose-6-P, and 50 mM P₁ (for assay conditions, see following section). The enzyme was found to be rather unstable, losing approximately one-half of the original activity following storage at refrigerator temperatures for 1 week.

**Phosphofructokinase Assay—**Erythrocyte P-fructokinase activity was measured by fructose 1,6-diphosphate formation following incubation of the enzyme in a 1.0 ml reaction mixture containing ATP, fructose-6-P, MgCl₂ (at concentrations equal to ATP additions), 50 mM Tris buffer at pH 8.0, and P₁ (as Na₂HPO₄) when indicated. Incubations were carried out for 15 min at 37°C. Reactions were terminated by immersion of the reaction tubes in a boiling water bath for 2 min. Fructose 1,6-diphosphate formation was determined on aliquots of the heat-inactivated solutions by fluorometric measure as described in an earlier section.

**RESULTS**

**Glucose Degradation and P₁—**Erythrocytes incubated in the presence of excess glucose in strongly buffered isotonic media utilize glucose and produce lactic acid at constant rates over extended periods of incubation (3). Comparison of glucose metabolism by these cells in the presence and absence of added P₁ shows several characteristic features which are depicted in Fig. 1. In accordance with the finding of others (2, 4) the rate of glucose utilization, as measured by disappearance of free glucose from the incubating mixture, is enhanced by the presence of P₁. P₁-enhanced glucose utilization rates are found to be maintained over prolonged incubation periods as illustrated. Measurement of lactic acid formation reveals an approximate stoichiometric conversion of the glucose only when the incubation is carried out in the absence of added P₁. Incubation with P₁ on the other hand, results in an enhanced utilization of glucose by the cells and an accompanying characteristic deficiency in lactic acid production.

**Effect of P₁ Concentration and pH on Glucose Degradation—**A comparison of the rates of glucose utilization and lactic acid production during the incubation of erythrocytes with a range of concentrations of P₁ is illustrated in Table I. With increasing P₁ concentrations glucose utilization was found to be continually enhanced. Lactic acid production, on the other hand, approached a maximum at rather low P₁ concentrations and accounted for only two-thirds of the glucose utilized at high P₁ levels.

Further information relating to the nature of the P₁ effect on glucose metabolism by the erythrocyte is summarized in Table II. The relative rates of glucose utilization and lactate production were compared at pH 7.2 and 7.8 in the presence and absence of added P₁. Stimulation of glucose utilization by P₁ was demonstrable at either pH. However, considerably less utilization occurred at the lower pH. Lactate to glucose ratios were found to be similarly reduced in the presence of P₁ at both pH 7.2 and 7.8, in spite of the decreased utilization of glucose by the cells at the lower pH. Therefore, the reduced lactate to glucose ratio induced by P₁ could not be simply attributed to differences in the rate of glucose utilization by the cells.

**Table I**

**Effect of P₁ on glucose utilization and lactate production by intact erythrocytes**

Washed cells were incubated in isotonic media at 37°C in 10% suspension (final concentration) in the presence of 0.05 M glycyglycine buffer at pH 7.8, glucose at 12 μmoles per ml of cells, phosphate as indicated, and sodium chloride as required. The pH was maintained within 0.15 unit over the entire incubation period.

<table>
<thead>
<tr>
<th>Concentration of added P₁</th>
<th>μmoles glucose utilized</th>
<th>μmoles lactate produced</th>
<th>Ratio of lactate to glucose</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>3.7</td>
<td>7.5</td>
<td>2.0</td>
</tr>
<tr>
<td>2</td>
<td>4.4</td>
<td>9.0</td>
<td>2.0</td>
</tr>
<tr>
<td>10</td>
<td>7.5</td>
<td>12.1</td>
<td>1.6</td>
</tr>
<tr>
<td>50</td>
<td>8.0</td>
<td>10.7</td>
<td>1.3</td>
</tr>
</tbody>
</table>

**Table II**

**Effect of pH and P₁ on glucose utilization and lactate production by intact erythrocytes**

Washed cells were incubated in isotonic media at 37°C in final 10% suspension (final concentration) in the presence of 0.05 M glycyglycine buffer at either pH 7.2 or 7.8, glucose at 12 μmoles per ml of cells, phosphate as indicated, and sodium chloride as required. The average hourly rates of glucose disappearance and lactate production were determined over a 4-hour incubation period.

<table>
<thead>
<tr>
<th>Concentration of added P₁</th>
<th>pH</th>
<th>Glucose utilized</th>
<th>Lactate produced</th>
<th>Ratio of lactate to glucose</th>
</tr>
</thead>
<tbody>
<tr>
<td>mM</td>
<td>μmoles/100 Hb/hr</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>7.2</td>
<td>1.6</td>
<td>2.9</td>
<td>1.8</td>
</tr>
<tr>
<td>25</td>
<td>7.2</td>
<td>4.9</td>
<td>7.3</td>
<td>1.5</td>
</tr>
<tr>
<td>10</td>
<td>7.8</td>
<td>3.2</td>
<td>6.0</td>
<td>1.9</td>
</tr>
<tr>
<td>25</td>
<td>7.8</td>
<td>8.0</td>
<td>9.8</td>
<td>1.2</td>
</tr>
</tbody>
</table>

**Fig. 1.** Effect of P₁ on glucose utilization and lactate production by the intact erythrocyte.
TABLE III
Cumulative changes in cellular phosphate concentrations accompanying glucose metabolism by intact erythrocyte in presence and absence of added inorganic phosphate

Washed cells were incubated in isotonic media at 37°C as 10% suspensions in 0.05 M glycylglycine buffer at pH 7.8, glucose at 12 μmoles per ml of cells, 0.05 M Pi (at pH 7.8) where indicated, and NaCl as required. Following 3 hours of incubation at 37°C, the cells were centrifuged, resuspended in fresh identical substrate media and incubated for a second 3-hour period.

<table>
<thead>
<tr>
<th>Incubation</th>
<th>Incubation time (hrs)</th>
<th>Cellular phosphates*</th>
<th>glucose utilized</th>
<th>lactate produced</th>
<th>Δf</th>
</tr>
</thead>
<tbody>
<tr>
<td>+P1</td>
<td>0</td>
<td>APi (7.6) (45)</td>
<td>17.4</td>
<td>26.5</td>
<td>-8.3</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>+47.4 +7.3</td>
<td>37.2</td>
<td>58.7</td>
<td>-21.7</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>+50.4 +18.1</td>
<td>6.4</td>
<td>15.1</td>
<td>+8.3</td>
</tr>
<tr>
<td>-P1</td>
<td>0</td>
<td>APi (7.6) (49)</td>
<td>13.3</td>
<td>27.5</td>
<td>+14.2</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>-5.2 -4.1</td>
<td>6.4</td>
<td>15.1</td>
<td>+9.3</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>-4.9 -0.5</td>
<td>13.3</td>
<td>27.5</td>
<td>+0.9</td>
</tr>
</tbody>
</table>

* The ΔPi and ΔP3 refer to cumulative changes in Pi and acid-soluble organic phosphates (P3) accompanying the incubations. The initial cellular Pi and P3 values are indicated in parentheses.
† The cumulative differences between the expected lactate (assuming total conversion of utilized glucose to lactate) and that actually produced are indicated in this column.

The possibility of P1-induced enhanced CO2 production (via the hexosemonophosphate pathway (17)) accounting for a significant fraction of erythrocyte glucose metabolism was examined. In the presence of P1 at concentrations effecting maximum stimulation of glucose utilization liberation of CO2 by erythrocytes was found to be small, representing <2% of the metabolized glucose carbon.

Changes in Cellular Phosphates—Incubation of erythrocytes with glucose and Pi results in cellular accumulation of both inorganic and organic phosphates as illustrated in the experiment summarized in Table III. Changes in cellular phosphates accompanying glucose metabolism in the absence of added Pi are also shown. Incubation with Pi resulted in a continuous accumulation of organic phosphate accompanying glucose degradation by the cells. Accumulation of organic phosphate was compared with glucose utilization and lactate production by the cells in the present study. As shown in the summarized results, accumulation of organic phosphate was found to approach closely that fraction of the metabolized glucose not appearing as lactate. Little change in the concentration of cellular organic phosphate occurred in the course of glucose metabolism by erythrocytes incubated in the absence of Pi.

It was clear from these findings that the presence of P1 induces (in some manner) an accumulation in the erythrocyte of one or more phosphorylated intermediate of glucose degradation.

Identification of Accumulating Intermediates—In order to delineate further the nature of the P1 effect on erythrocyte glycolysis, identification of the accumulating phosphorylated intermediate or intermediates was systematically sought. Although the erythrocyte is known to contain unusually large amounts of glucose 1,6-diphosphate and 2,3-diphosphoglycerate (18), analyses failed to reveal significant accumulation of either in the course of glucose degradation by erythrocytes in the presence of P1. Systematic analyses of other metabolic intermediates were subsequently carried out resulting in the findings summarized in Fig. 2. Among the variety of intermediates examined, accumulation of dihydroxyacetone-P, fructose 1,6-diphosphate, and α-glycerol-P was found. The presence in the erythrocyte of α-glycerol-P as an intermediate of glucose degradation has apparently not been previously described. A
plot of the sums of the accumulated dihydroxyacetone-P, α-glycerol-P, and fructose 1,6-diphosphate is shown. Values representing the calculated differences between the "theoretical" (i.e., a yield of 2 moles of lactate per mole of glucose utilized) and actual lactate yields obtained in this particular experiment are also shown. A comparison of these two latter plots reveals a nearly stoichiometric accounting of the "missing" lactate by the three accumulated intermediates.

Accumulation of fructose 1,6-diphosphate accompanying P_i-induced enhancement of glucose utilization implicates the enzyme, P-fructokinase, as a probable primary site of P_i activation. Since a reversal of ATP-induced inhibition of P-fructokinase activity by P_i (among other agents) has been described (5, 6) with various enzyme preparations, study of the purified erythrocyte enzyme was undertaken.

Relationship Between P_i and Erythrocyte P-fructokinase Activity—Erythrocyte P-fructokinase was prepared in purified form (see "Experimental Procedure") and employed in these studies. The specific characteristics of the inhibition and activation of the enzyme were examined. Incubation of the enzyme with substrates (i.e., ATP and fructose-6-P) at concentrations approximating their normal cellular levels resulted in inhibition with little detectable fructose 1,6-diphosphate formation. On the other hand, addition of excess P_i to such reaction mixtures resulted in an activation, followed by synthesis of fructose 1,6-diphosphate. These findings are illustrated in the experiment summarized in Fig. 3 in which ATP was added at 1 mM concentration (approximate normal cellular level (3)) and fructose-6-P was varied over a range of concentrations (again approximating a range of normal cellular levels). Synthesis of fructose 1,6-diphosphate was shown in this study only on addition of P_i, and was found to be linearly proportional to the fructose-6-P concentrations employed. The relationship between P-fructokinase activation and P_i concentration is illustrated in the experiment shown in Fig. 4. At the substrate concentrations employed, maximal activation of the inhibited enzyme was induced in the presence of 20 mM P_i. Measurable activation occurred at all lower concentrations of P_i tested in the manner illustrated. The relationship between P-fructokinase activation by P_i and ATP concentration is illustrated in the experiment summarized as Fig. 5. In the absence of added P_i, little measurable fructose 1,6-diphosphate formation occurred at ATP concentrations of 1 mM and higher. Enzyme activity was demonstrable at all lower ATP concentrations tested as shown. Addition of P_i at 1, 5, and 25 mM to a similar series of reaction mixtures resulted in increased fructose 1,6-diphosphate formation to the extent illustrated.

Further study of the specific kinetic properties of the erythrocyte P-fructokinase (or P-fructokinases (19)) is being separately undertaken.

DISCUSSION

The experiments described in the present report implicate the enzyme, P-fructokinase, as a primary site of P_i action in effecting stimulation of glucose utilization by the intact human erythrocyte. The relationship between the apparent dual role of P_i for regulating erythrocyte hexokinase (2) and P-fructokinase activities remains to be assessed further. It is clear, however, that entry of glucose into the Embden-Meyerhof pathway and conversion to fructose 1,6-diphosphate occur at accelerated rates as a consequence of P_i stimulation. On the other hand, lactate production is not equally accelerated, with accumulation of fructose 1,6-diphosphate, dihydroxyacetone-P, and α-glycerol-P accounting stoichiometrically for the differences in rates. From the nature of the accumulation products, lactate production is clearly limited at a step subsequent to triose-P formation. Since enhanced glucose degradation accompanying P_i stimulation presumably results in the maintenance of high levels of ATP, the availability of ADP could limit the rate of lactate formed. Also, since our studies were carried out without addition of potassium to incubating media, ADP generation (from ATP) coupled with normal active cation transport mechanism is presumably excluded (e.g., see Reference 20), thereby further limiting the availability of ADP. Although P_i inhibition of a step subse-
quent to triose-P formation might limit lactate production, we have not as yet systematically examined this possibility. Evidence suggesting that DPN is not a factor limiting lactate production in the intact erythrocyte has been obtained and will be considered further elsewhere.

Examination of the kinetic properties of the purified erythrocyte P-fructokinase reveals a pattern of activation by P_i which is generally consistent with the view that the enzyme is a primary site of P_i action in the stimulation of glucose utilization by the intact erythrocyte. Thus, activation of erythrocyte P-fructokinase by P_i can be demonstrated under simulated cellular conditions with respect to substrate concentrations and pH. The extent to which other known activators of P-fructokinases (e.g., see References 5, 6, and 19) are involved in the overall cellular regulation of the erythrocyte enzyme remains to be assessed.

Further information relating to the sensitivity of P-fructokinase as a regulatory center of glucose degradation in the intact erythrocyte is contained in a recent report (21).

SUMMARY

The nature of inorganic phosphate-induced stimulation of glucose utilization by intact erythrocytes was examined. Cellular accumulations of fructose 1,6-diphosphate, dihydroxyacetone phosphate, and α-glycerophosphate were found to accompany enhanced glucose utilization resulting from stimulation by inorganic phosphate. From the nature of the accumulation products and examination of the kinetic properties of purified erythrocyte phosphofructokinase, the enzyme was considered to represent a primary site of inorganic phosphate activation, leading to enhanced glucose utilization by the intact erythrocyte.

REFERENCES

Inorganic Phosphate and Enhanced Glucose Degradation by the Intact Erythrocyte
K. K. Tsuboi and K. Fukunaga