Erythrocyte phosphofructokinase (PFK) is one of the major regulators of glycolysis. Adenosine triphosphate (ATP) serves as an inhibitor as well as a substrate. Inhibition by ATP reduces the affinity of the enzyme for its other substrate, fructose-6-phosphate (F-6-P). Considering physiological concentrations of ATP and F-6-P in erythrocytes, the enzyme functions at only 0.1% of its capacity, which corresponds to about 10% of the glycolytic rate.

Although this inhibition by ATP is mediated by ATP-Mg\(^{2+}\) complex, which is less inhibitory than ATP, the normal intracellular activity of PFK is largely dependent on the action of positive effectors, including adenosine monophosphate (AMP), adenosine diphosphate (ADP), fructose-1,6-bisphosphate (F-1,6-P\(_2\)) and glucose-1,6-bisphosphate (G-1,6-P\(_2\)).

Fructose-2,6-bisphosphate (F-2,6-P\(_2\)) is present in the liver and is the most potent effector of hepatic PFK by releasing the ATP inhibition. Although human erythrocyte PFK was also strongly activated by F-2,6-P\(_2\), the absence of this metabolite in human erythrocytes has been reported.

The standard method to assay intracellular F-2,6-P\(_2\) requires heating the tissue or cells at 80°C in an alkaline solution. Our preliminary experiment showed that it was extremely difficult to eliminate hemoglobin using this method, which, to a great extent, interfered with the F-2,6-P\(_2\) assay system. In addition, chicken erythrocytes recently have been reported to contain this bisphosphorylated metabolite. Therefore, these findings prompted us to reevaluate F-2,6-P\(_2\) in human erythrocytes.

To test the possibility of its existence, we examined the enzyme’s ability to synthesize F-2,6-P\(_2\), fructose-6-phosphate,2-kinase (F-6-P,2-kinase) in the partially purified hemolysate and the effect of this metabolite on the rate-limiting enzymes.

### MATERIALS AND METHODS

All reagents used were analytical grade. Commercial F-2,6-P\(_2\) from Sigma (St Louis) was found to be contaminated with 0.1 to 0.4% F-1,6-P\(_2\). Blood from a healthy volunteer was drawn into heparin and freed of white cells and platelets by passing it through a cellulose column. After washing with 0.85% NaCl, the cells were lyzed in the 10 mmol/L phosphate buffer, pH 7.0. The stroma-free hemolysate was obtained by centrifugation and applied to DE 52 batchwise chromatography to remove hemoglobin. The eluate was fractionated by adding ammonium sulfate. The fractions of 35% to 60% and 60% to 80% saturation were subjected to the following study after dialysis against 20 mmol/L HEPES buffer, pH 7.4.

F-6-P,2-kinase activity was measured as described elsewhere by assaying F-2,6-P\(_2\) synthesized from F-6-P in the presence of ATP. The reaction mixture comprised 20 mmol/L HEPES, pH 7.4, 10 mmol/L MgCl\(_2\), 1 mmol/L EDTA, 1 mmol/L dithiothreitol, 5 mmol/L ATP, various concentrations of F-6-P, and the enzyme. After 20 minutes' incubation at 37°C the reaction was terminated by adding 100 μL of 0.5 N NaOH followed by heating at 80°C for 20 minutes. After cooling and centrifuging, half of the supernatant was neutralized with acetic acid in the presence of 20 mmol/L HEPES. The formation of F-2,6-P\(_2\) was determined by measuring its stimulatory effect on pyrophosphate-phosphofructokinase (PPi-PFK) in the presence of 0.5 mmol/L pyrophosphate and 1 mmol/L F-6-P. Since F-2,6-P\(_2\) is easily destroyed under mild acid conditions, 0.5 N HCl was added to the other half of the supernatant. After standing at 25°C at a pH between 1 and 2 for ten minutes, the acid-treated supernatant was neutralized with NaOH and used as a control.

PFK and pyruvate kinase (PK) were assayed by the method of Beutler. Hexokinase was assayed in the absence and presence of its effectors by the previous method.

### RESULTS

Figure 1 shows the formation of F-2,6-P\(_2\) from F-6-P in the presence of the various fractions prepared from hemolysate by ammonium sulfate fractionation. In the fraction of 35% to 60% saturation, F-2,6-P\(_2\) synthesis was seen, which was determined by the stimulation of PPI-PFK. In the control, stimulation was completely suppressed by preliminary treatment of the extract with HCl at pH 1 to 2. On the other hand, the fraction of 60% to 80% saturation showed no F-2,6-P\(_2\) formation.

Figure 2 shows the effect of F-2,6-P\(_2\) on PFK activity. Km for F-6-P in the absence and presence of 1 mmol/L F-2,6-P\(_2\) was 1.7 mmol/L and 0.7 mmol/L, respectively.

Figure 3 shows PFK activity as the function of F-2,6-P\(_2\) concentration. With the concentrations of both 0.5 mmol/L (Fig 3A) and 1.0 mmol/L F-6-P (Fig 3B), ~90% maximum activation was obtained at 10^{-6} mol/L F-2,6-P\(_2\) and the half-maximum activation was at 10^{-7} mol/L F-2,6-P\(_2\).

Table 1 shows the effect of F-2,6-P\(_2\) on hexokinase activity in the absence and presence of various inhibitors. No effect was detected.
F-2,6-P₂ is acid-labile and alkaline-stable. The standard method for the determination of the glycolytic intermediates in red cells, which requires acid-treatment of the cells, is not used. On the other hand, alkaline-treatment at 80°C is not applicable either, because hemoglobin remaining after the treatment strongly interfered with the assay system for F-2,6-P₂ (data not given). Therefore, our present work has been to test the possibility of its existence, namely the demonstration of the enzyme activity to synthesize F-2,6-P₂ and the activation of PFK by this metabolite.

As shown in Fig. 1, the fraction of 35% to 60% saturation was capable of synthesizing F-2,6-P₂ from F-6-P and ATP. The determination of this bisphosphorylated metabolite was based on its ability to stimulate PPi-PFK. Although it is not a direct determination, it is an established method. Well-known activators for PPi-PFK, such as ADP, AMP, F-6-P, F-1,6-P₂ and G-1,6-P₂, are all acid-stable, while F-2,6-P₂ is acid-labile and alkaline-stable.

**DISCUSSION**

F-2,6-P₂ is acid-labile and alkaline-stable. The standard method for the determination of the glycolytic intermediates in red cells, which requires acid-treatment of the cells, is not used. On the other hand, alkaline-treatment at 80°C is not applicable either, because hemoglobin remaining after the treatment strongly interfered with the assay system for F-2,6-P₂ (data not given). Therefore, our present work has been to test the possibility of its existence, namely the demonstration of the enzyme activity to synthesize F-2,6-P₂ and the activation of PFK by this metabolite.

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**Table 1. Effect of F-2,6-P₂ on HK Activity in the Presence of the Inhibitors**

<table>
<thead>
<tr>
<th>Additives</th>
<th>F-2,6-P₂ (μmol/L)</th>
<th>None (μmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>G-6-P (25 μmol/L)</td>
<td>36.0 ± 1.1</td>
<td>38.3 ± 2.1</td>
</tr>
<tr>
<td>ADP (3.0 mmol/L)</td>
<td>65.0 ± 4.0</td>
<td>69.1 ± 0.9</td>
</tr>
<tr>
<td>G-1,6-P₂ (200 μmol/L)</td>
<td>80.7 ± 3.4</td>
<td>84.6 ± 2.8</td>
</tr>
</tbody>
</table>

Assay mixture for HK consisted of 50 mmol/L Tris-HCl, pH 8.0, containing 5 mmol/L MgCl₂, 0.2 mmol/L NADP, 1.0 mmol/L ATP, 0.1 U/mL G-6-PD and 50 μmol/L glucose (Km of HK for glucose) in the absence and presence of the physiological inhibitors. The mean values (±SD) were obtained from four to six independent experiments. No effects were observed even at 5 mmol/L glucose (data not given).
acid-labile and alkaline-stable. Up to now there have been no other compounds possessing these two properties.\textsuperscript{10} In the control of the 35\% to 60\% saturation fractionation, F-2,6-P\_2 was completely destroyed by the acid-treatment. These results indicate that human red cells contain F-6-P,2-kinase activity.

F-2,6-P\_2 has been reported to be an effector of several enzymes in glycolysis, glycogenolysis, and gluconeogenesis.\textsuperscript{12,13} However, erythrocytes lack the latter two pathways. Of the eleven enzymes in the glycolytic pathway, three appear to be particularly important: hexokinase, PFK, and PK. Hexokinase is the least active enzyme in the series and, therefore, often rate-limiting. Hexokinase is partially inhibited by some glycolytic intermediates and its activators act by releasing the inhibition.\textsuperscript{7} As shown in Table 1, F-2,6-P\_2 had no effect on the hexokinase activity even in the presence of these inhibitors such as G-6-P, G-1,6-P\_2 and ADP.

PK from rat liver (L-type PK) has been reported to be activated by F-2,6-P\_2.\textsuperscript{14} Since erythrocyte PK is the same genetic product as L-type PK and has immunologically and kinetically similar properties, the activation of erythrocyte PK similar to that of L-type PK had been expected. However, as we show, erythrocyte PK was activated not only by nontreated commercial F-2,6-P\_2, but also by acid-treated F-2,6-P\_2, indicating that this activation was not due to authentic F-2,6-P\_2. As commercial F-2,6-P\_2 contains a minute amount of F-1,6-P\_2, the activation of L-type PK as well as erythrocyte PK may be explained by the contaminant.

F-2,6-P\_2 has been known to activate erythrocyte PFK.\textsuperscript{4} However, the concentration for the half-maximum activation obtained here (10\textsuperscript{-6} mol/L) was lower than that of the previous report (10\textsuperscript{-4} mol/L)\textsuperscript{4} and 10\textsuperscript{-6} mol/L F-2,6-P\_2 was high enough to activate PFK up to 90\% of its maximum effect (Fig 3). F-2,6-P\_2 used here was also contaminated with F-1,6-P\_2, but the concentration of the contaminant was too low to affect PFK activity.

From these results, it is strongly suggested that F-2,6-P\_2 may be one of the intracellular constituents and may contribute to the regulation of PFK activity, and consequently, glycolysis in human erythrocytes.

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Fructose-6-phosphate,2-kinase activity in human erythrocytes
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