Regulatory Mechanisms in Carbohydrate Metabolism

VII. HEXOKINASE AND PHOSPHOFRUCTOKINASE*

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Inhibition of mammalian hexokinase by glucose 6-phosphate was first reported by Weil-Malherbe and Bone (1) and studied by Crane and Sols (2). An inhibition of rabbit muscle phosphofructokinase by excess adenosine triphosphate was observed by Lardy and Parks (3) and was shown to be reversed by a number of intracellular components by Passonneau and Lowry (4). Similar findings were reported on the enzyme from liver fluke (5), heart muscle (6), and yeast (7).

A possible role for glucose-6-P inhibition of hexokinase in the Pasteur effect was proposed many years ago (8), but it was realized that by itself it did not represent a sufficient control mechanism. When the inhibition of P-fructokinase by ATP was shown to be reversed by inorganic orthophosphate (4), the possibility of a sequential control mechanism was considered and a reinvestigation of the Pasteur effect in reconstructed systems was initiated. While this investigation was in progress, a stimulation of hexokinase by Pi (9) was shown to be due to a reversal of the glucose-6-P inhibition (10).

Previous experiments (11) on reconstructed systems were performed with crystalline yeast hexokinase. Since this enzyme does not exhibit a product inhibition by glucose-6-P, it was necessary to undertake the purification and study of a suitable mammalian enzyme. It is the purpose of this paper to describe and a reinvestigation of the Pasteur effect in reconstructed systems was initiated. While this investigation was in progress, a stimulation of hexokinase by Pi (9) was shown to be due to a reversal of the glucose-6-P inhibition (10).

Analytical Methods

Spectrophotometric assays were all performed in a Beckman model DU spectrophotometer equipped with a Gilford automatic sample changer and recorder.

Definitions of Unit and Specific Activity—A unit of activity is defined as the amount of enzyme that catalyzes the formation of 1 µmole of product per min under the specified assay conditions. Specific activity is expressed as units per mg of protein.

Assay of Hexokinase Activity

Assay I (Spectrophotometric Determination of Glucose-6-P)—The assay mixture contained in a final volume of 1 ml: 100 mM Tris-Cl (pH 7.6), 5 mM MgCl₂, 5 mM ATP, 1 mM glucose, 0.4 mM TPN, and 0.3 unit of glucose-6-P dehydrogenase. The reaction was initiated by the addition of hexokinase. The rate of the reaction was calculated from the increase in absorbance at 340 µm at a linear portion of the curve. This assay was used during the purification of the enzyme.

Assay II (Isolation and Determination of 14C-Glucose-6-P)—The assay method was the same as that described by Rose and O'Connell (14), except that in most experiments, 0.5-ml aliquots of the eluate from the Dowex 1 column were counted directly in a scintillation counter according to Wu (15).

Assay III (Spectrophotometric Determination of ADP)—The reaction mixture contained in a final volume of 1 ml: 100 mM Tris-Cl (pH 7.6), 5 mM MgCl₂, 1 mM P-enolpyruvate, 1 mM glucose, 1 mM ATP, 0.15 mM DPNH, 1.2 units of pyruvate kinase, and 1.5 units of lactate dehydrogenase. The reaction was initiated by the addition of hexokinase, and the decrease in absorbance at 340 µm was recorded. A control without glucose served to measure ATPase activity.

Standard Assay of P-fructokinase Activity—P-fructokinase activity was determined by measuring triose-P formation as described by Wu and Racker (16), except that 100 mM Tris-
HCl (pH 8.0), 1.5 mM fructose-6-P, 1 mM ATP, 5 mM MgCl₂,
and 100 mM 2-mercaptoethanol were used. The enzyme was
incubated just before assay in the mixture of 50 mM Tris (pH 7.6),
3 mM EDTA, and 100 mM 2-mercaptoethanol. The crystalline
auxiliary enzymes contained sufficient ammonium sulfate to
saturate P-fructokinase with NH₄⁺.

The inhibition of P-fructokinase by ATP was determined as
follows.

**Assay A**—The assay mixture contained in a final volume of 1
ml: 50 mM imidazole HCl (pH 7.1), 5 mM MgCl₂, 60 mM fructose-
6-P, 100 mM 2-mercaptoethanol, 0.2 mM DPNH, three levels of
ATP concentrations, 0.5, 2, and 4 mM, 0.8 unit of aldolase, 1.5
units of fructose-1,6-P₂ isomerase, and 0.5 unit of α-glycerol-P dehy-
drogenase. The reaction was initiated by the addition of P-
fructokinase, and the decrease in absorbance at 340 nm was
recorded.

**Assay B**—This assay was essentially the same as in Assay A
except that 50 mM Tris buffer (pH 7.4), 1 mM NH₄Cl, and 100
mM 2-mercaptoethanol were used. Moreover, all the enzymes
except P-fructokinase were dialyzed overnight at 2° against
large volumes of 1.8 mM EDTA (pH 7.4).

**Determination of Protein**—Protein content was determined
according to Sutherland et al. (17).

**Preparations**

**Purification of Ascites Tumor Hexokinase**

**Step 1. Preparation of Acetone Powder**—Ehrlich tetraploid
ascites tumor cells were collected 7 to 8 days after transplanta-
tion in 2 volumes of a solution containing 0.25 M mannitol and
5 mM EDTA (pH 7.4). The cells were centrifuged at 600 × g
for 10 min and washed once with 5 volumes of mannitol-EDTA.
The packed cells, approximately 80 ml, were blended for 30 sec
with a Waring Blender in 600 ml of acetone at −15°. The cell
suspension was collected in a cold room on a (15-cm diameter)
Buchner funnel and washed twice with 250 ml each of acetone
at −10°. The acetone was removed from the powder under
reduced pressure at room temperature. All subsequent opera-
tions were performed at 0°C unless otherwise stated.

**Step 2. Extraction of Acetone Powder**—The acetone powder (4 g)
was suspended in 80 ml of 10 mM potassium phosphate buffer
(pH 7.5), containing 5 mM EDTA (pH 7.2), and 20 mM 2-
mercaptoethanol. The suspension was allowed to stand at 0°C
for 20 min with occasional stirring to break up the clumps and
was then centrifuged in a Spincel preparative ultracentrifuge at
30,000 × g for 10 min.

**Step 3. Treatment with Protamine Sulfate**—To the supernatant
solution (65 ml) were added 16 ml of a 2% solution of protamine
sulfate (pH 6.8), and after the mixture had stood for 5 min at
0°C, it was centrifuged at 30,000 × g for 10 min.

**Step 4. Fractionation with Ammonium Sulfate (I)**—To 75 ml
of the supernatant solution from Step 3 we added an equal
volume of saturated ammonium sulfate solution (pH 8.0) and
0.2 ml of 1 M 2-mercaptoethanol. The mixture was allowed
to stand for 15 min and centrifuged for 10 min at 30,000 × g.
The precipitate was dissolved in 5 ml of the buffer solution used
in Step 2.

**Step 5. Treatment with Trypsin and Chymotrypsin**—To 7.1
ml of the solution from Step 4 we added 0.36 ml of 1 M tri-
ethanolamine-HCl (pH 8.0), 0.7 ml of 1 M glucose, and 0.9 ml
each of 1% chymotrypsin and 1% trypsin. The mixture was
incubated for 1 hour at 37°. Proteolysis was terminated by the
addition of 0.25 ml of diisopropyl fluorophosphosphate solution
which was prepared immediately before use by diluting 10 µl
of the compound in 3 ml of water. If the digested enzyme solu-
tion was cloudy, it was clarified by centrifugation.

**Step 6. Fractionation with Ammonium Sulfate (II)**—Ammoni-
um sulfate (2.18 g) was added to 9.7 ml of the supernatant
solution from Step 5, and the mixture adjusted to pH 7.0 with
15 N NH₄OH. The solution was centrifuged at 30,000 × g for
5 min, and the precipitate was discarded. To the supernatant
solution, 1.8 g of ammonium sulfate were added, and the pH
was adjusted to 7.0 with 15 N NH₄OH. The solution was allowed
to stand for 15 min and centrifuged for 10 min at 30,000 × g.
The precipitate was dissolved in 1 ml of 5 M potassium phos-
phate (pH 7.4) containing 5 mM EDTA, 20 mM 2-mercaptoethanol,
and 10 mM glucose. The enzyme was usually found in Fractions 11 to 15 which were
combined.

**Step 7. Purification on Sephadex G-75**—The enzyme solution
from Step 6 was placed at room temperature on a Sephadex
G-75 column (1.8 × 21 cm), previously equilibrated with the
weakly buffered solution used in Step 6, to which 10 mM glucose
was added. The enzyme was eluted from the column with the
same solution, and 1-ml fractions were collected. The enzyme
usually appeared in Fractions 14 to 17, which were combined.

**Step 8. DEAE-cellulose Chromatography**—A DEAE-cellulose
column (1 × 17 cm) was packed with pressure and equilibrated
with the same solution used in Step 7. The pooled enzyme was
placed on the column and, after washing with 20 to 30 ml of
the same buffer, hexokinase was eluted in 1-ml fractions with a
solution containing 30 mM potassium phosphate (pH 7.4), 5
mM EDTA, 20 mM 2-mercaptoethanol, and 10 mM glucose.
The enzyme was usually found in Fractions 11 to 15 which were
combined.

The results of this purification procedure are summarized in
**Table I**.

**Purification of P-fructokinase**

**Step 1. Ammonium Sulfate (I)**—The procedure for the prep-
oration of rabbit muscle extract, precipitation of the enzyme
and extraction with 40% saturated ammonium sulfate have been
described previously (11).

**Table I**

<table>
<thead>
<tr>
<th>Fractionation step</th>
<th>Units/ml</th>
<th>Total units</th>
<th>Protein</th>
<th>Specific activity</th>
<th>Yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>2. Extract</td>
<td>1.2</td>
<td>78</td>
<td>14.2</td>
<td>0.084</td>
<td>100</td>
</tr>
</tbody>
</table>
| 3. Protamine sulf-
| ate            | 1.86     | 64          | 5.5     | 0.16              | 83    |
| 4. Ammonium sulf-
| ate (I)        | 8.4      | 60          | 19      | 0.43              | 77    |
| 5. Trypsin-chymo-
| trypsin treat-
| ment           | 3.6      | 35          | 21      | 1.1               | 30    |
| 6. Ammonium sulf-
| ate (II)       | 23       | 23          | 21      | 1.7               | 23    |
| 7. Sephadex chro-
| matography     | 3.9      | 23          | 21      | 1.7               | 29    |
| 8. DEAE-cellulose
| chromatography | 2.1      | 14          | 0.23    | 9.1               | 17    |
TABLE II
Purification of phosphofructokinase from rabbit muscle

<table>
<thead>
<tr>
<th>Steps</th>
<th>Volume</th>
<th>Units/ml</th>
<th>Total units</th>
<th>Protein (mg/ml)</th>
<th>Specific activity</th>
<th>Yield %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Muscle extract* ...</td>
<td>800</td>
<td>22.6</td>
<td>18,000</td>
<td>16</td>
<td>1.4</td>
<td>7%</td>
</tr>
<tr>
<td>1. Extract of ammonium sulfate (II) ...</td>
<td>1,000</td>
<td>36</td>
<td>36,000</td>
<td>11</td>
<td>3.3</td>
<td>100</td>
</tr>
<tr>
<td>2. Ammonium sulfate (II) ...</td>
<td>23</td>
<td>1,350</td>
<td>31,200</td>
<td>226</td>
<td>6.0</td>
<td>88</td>
</tr>
<tr>
<td>3. First Sephadex ...</td>
<td>11.5</td>
<td>1,140</td>
<td>13,100</td>
<td>23</td>
<td>50</td>
<td>36</td>
</tr>
<tr>
<td>4. Second Sephadex ...</td>
<td>2</td>
<td>3,880</td>
<td>7,760</td>
<td>17</td>
<td>228</td>
<td>22</td>
</tr>
</tbody>
</table>

* Preparation from one rabbit.

TABLE III
Effect of P_i on inhibition of partially purified hexokinases by glucose-6-P

<table>
<thead>
<tr>
<th>Source of hexokinase</th>
<th>Glucose-6-P concentration of:</th>
<th>Δ Absorbance/min at P_i concentration of:</th>
<th>Inhibition of P_i concentration of:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 mm</td>
<td>5 mm</td>
<td>10 mm</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>%</td>
</tr>
<tr>
<td>Brain</td>
<td>0</td>
<td>0.041</td>
<td>0.042</td>
</tr>
<tr>
<td>0.046</td>
<td>0.034</td>
<td>0.040</td>
<td>0.040</td>
</tr>
<tr>
<td>0.184</td>
<td>0.021</td>
<td>0.032</td>
<td>0.032</td>
</tr>
<tr>
<td>Ascites tumor</td>
<td>0</td>
<td>0.066</td>
<td>0.068</td>
</tr>
<tr>
<td>0.046</td>
<td>0.039</td>
<td>0.049</td>
<td>0.049</td>
</tr>
<tr>
<td>0.184</td>
<td>0.013</td>
<td>0.023</td>
<td>0.023</td>
</tr>
</tbody>
</table>

Step 2. Ammonium Sulfate (II)—To 70 ml of the solution obtained from Step 1, 12 ml of saturated ammonium sulfate were added, and the mixture was allowed to stand overnight. The precipitate was centrifuged at 30,000 × g for 10 min and dissolved in a buffer containing 50 mM potassium phosphate (pH 7.5), 5 mM EDTA, and 50 mM 2-mercaptoethanol.

Step 3. Sephadex Chromatography—The enzyme solution (23 ml) from Step 2 was placed on a Sephadex G-200 column (4.5 × 40 cm), which had been equilibrated with the buffer used in Step 2, to which 0.4 M ammonium sulfate was added. The enzyme was eluted with the same mixture and 3-m1 fractions were collected. The enzyme was collected in a volume of 145 ml after a void volume of 175 ml. To the eluate, sufficient solid ammonium sulfate was added to 38% saturation, and the pH was adjusted to 7.5 with 15 N NHOH. After centrifugation, the precipitate was dissolved in a minimal volume of the buffer used in Step 2, and sufficient ammonium sulfate was added to bring the saturation to 60%.

The enzyme was stored as the ammonium sulfate precipitate and was used for most experiments at this state of purity. The specific activity of different preparations at this step varied from 45 to 110.

Step 4. Second Sephadex—One preparation, however, was purified further by centrifuging the enzyme suspension of Step 3, dissolving the precipitate in a small volume of the buffer used in Step 2, and repeating the chromatography on the Sephadex column as in Step 3. The results of this purification procedure are summarized in Table II.

RESULTS

General Properties of Hexokinase—After DEAE-cellulose chromatography, the enzyme was purified about 100-fold compared to the crude extract and contained no detectable amounts of glucose-6-P dehydrogenase, glucose-6-phosphatase or ATPase. The purified enzyme was unstable in the absence of glucose when stored at 0°, −20°, or −70°. In the presence of 10 mM glucose, however, the enzyme was stable at 0° for at least 2 months with significant loss of activity. In the absence of glucose, the enzyme was stable at 38° at pH values between 6.8 to 7.5 for at least 1 hour but lost about 75% of the activity at pH values of 5.5 and 9.0.

Inhibition of Hexokinase by Glucose-6-P and Reversal by P_i—As shown in Table III, the partially purified hexokinase from brain as well as ascites tumor exhibited a pronounced inactivation by glucose-6-P, which was partially counteracted by P_i. In order to explore the possibility that quantitative differences in the susceptibility of the hexokinase to inhibition and in the reversal of inhibition by P_i may account for the high lactate production in tumor cells, we performed a comparative study with several crude hexokinase preparations. It can be seen from Table IV that the hexokinase in ascites tumor extracts and mouse brain homogenates was somewhat less susceptible to glucose-6-P inhibition and more readily reversed by P_i than the hexokinase in the red blood cell hemolysate. Yeast hexokinase (either crystalline or in a crude extract) was not inhibited by glucose-6-P under these experimental conditions.

The crude hexokinase from ascites tumor was less susceptible to glucose-6-P inhibition than the purified enzyme from the same source. The difference may be caused by compounds present in the crude extract which, similar to P_i, release glucose-6-P inhibition.

Attempts to Modify Product Inhibition of Hexokinase—Various attempts were made to modify the glucose-6-P inhibition, including heating at 53° and 65° in the presence of glucose-6-P, ATP, glucose, incubation with Hg2+ or Ag+, and proteolytic digestion with trypsin, chymotrypsin, or Nagarse in the presence and absence of glucose. None of these procedures altered the degree of inhibition of hexokinase by glucose-6-P.

Effect of ADP—As shown in Fig. 1, ADP appeared to be a competitive inhibitor of ATP, and the K_i was estimated as approximately 5.1 × 10^{-4} M. This competitive inhibition is in agreement with the results obtained with brain hexokinase by Sols and Crane (19), but differs from those of Fromm and Zwede (20). P_i, up to 10 mM had no effect on the ADP inhibition. The K_i for ATP was estimated to be 4.3 × 10^{-4} M.

Reversibility of Hexokinase Reaction and Partial Reactions—It can be seen from Table V that the reaction catalyzed by yeast hexokinase was readily reversible in confirmation of the results obtained by Gamble and Najjar (21) and Kaufman (22).
contrast, the purified ascites tumor enzyme tested at the same activity level neither catalyzed the reversal nor a partial exchange reaction. Since this difference in the properties of the two hexokinase preparations was thought to be due to the high susceptibility of the mammalian enzyme to glucose-6-P inhibition, attempts were made to demonstrate a reversal at much lower concentrations of glucose-6-P (0.58 mM). Thus far, these experiments have not yielded any indication for the reversibility of the reaction.

**General Properties of P-fructokinase**

The enzyme stored as an ammonium sulfate precipitate lost 10 to 20% activity in 1 month. The enzyme was found to be very sensitive to heavy metals such as Ag⁺ and Hg++. For example, in the presence of 10⁻⁶ M AgNO₃ or Ag₂SO₄ the enzyme (1 µg of protein per ml) was completely inactive.

The enzyme was unstable in dilute solution. At a protein concentration of 0.8 µg per ml, it lost 85% of its activity in 10 min at room temperature. Under these conditions, 1 mM glucose-6-P stabilized the enzyme, whereas in the presence of 5 mM ATP about 42% of the activity was lost; 10 mM Mg²⁺ had no effect.

The molecular weight of the enzyme was approximately 40,000 according to sucrose density gradient centrifugation performed as described by Martin and Ames (23).

**Stimulation by NH₄⁺ and 2-Mercaptoethanol**—A lack of proportionality was observed between activity and amount of protein with highly purified preparations of P-fructokinase. This was traced to the presence of variable amounts of NH₄⁺, which markedly stimulated the activity of this enzyme as shown in the following table.

<table>
<thead>
<tr>
<th>Glucose-6-P</th>
<th>F₁</th>
<th>Inhibition of hexokinase</th>
</tr>
</thead>
<tbody>
<tr>
<td>mM</td>
<td></td>
<td>Ascites tumor</td>
</tr>
<tr>
<td>——</td>
<td>——</td>
<td>——</td>
</tr>
<tr>
<td>0.1</td>
<td>0</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td>0.2</td>
<td>0</td>
<td>37</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td>0.5</td>
<td>0</td>
<td>74</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>57</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>39</td>
</tr>
<tr>
<td>1</td>
<td>0</td>
<td>89</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>79</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>72</td>
</tr>
</tbody>
</table>

**Fig. 1. Inhibition of hexokinase by ADP.** Assay I was used in this experiment in the presence of variable concentrations of ATP and ADP as indicated. Purified ascites tumor hexokinase (0.013 unit) was added to initiate the reaction.

**TABLE IV**

Release of glucose-6-P inhibition by P₁

Assay II and the indicated concentrations of glucose-6-P and P₁ were used. Hexokinase was added as follows: 0.029 unit of ascites homogenate, 0.02 unit of mouse brain homogenate, and 0.01 unit of red cells.

For the preparation of the brain homogenate, one mouse brain, weighing about 500 mg, was homogenized with a Potter-Elvejem homogenizer in 4 ml of 0.95 M mannitol-EDTA (1 mm), and centrifuged at 500 x g for 15 min. The supernatant fluid was used as a source of brain hexokinase. For the preparation of the ascites tumor homogenate, the cells were harvested 7 to 8 days after inoculation, washed twice with 4 volumes of 0.25 M mannitol-EDTA (1 mm), and suspended in 4 volumes of mannitol-EDTA. The suspension was then exposed to sonic oscillation for 5 min in a Raytheon sonic oscillator and filtered at 0° through Whatman No. 1 filter paper. For the preparation of the red blood cell hemolysate, heparinized human blood was obtained from a local blood bank. The cells were separated by centrifugation at 100 x g for 15 min and washed 3 times with 2 volumes of 0.25 M mannitol-EDTA (1 mm). To the washed red cell, an equal volume of H₂O was added. The solution was frozen quickly and thawed three times.
FIG. 2. Stimulation of P-fructokinase by NH₄⁺. The standard assay for P-fructokinase activity was used except that variable concentrations of NH₄Cl were added to the reaction mixture. For this experiment, the auxiliary enzymes were dialyzed overnight against 1.8 mM EDTA. P-fructokinase, which was kept in 2 M ammonium sulfate, was centrifuged and the supernatant solution was removed by decanting and by wiping the inner wall of the centrifuge tube with a piece of filter paper. The precipitate was then dissolved in a solution containing 50 mM Tris (pH 7.6), 1 mM EDTA, and 100 mM 2-mercaptoethanol. Immediately before use, P-fructokinase was diluted 1000-fold in the same solution, and 10 µl of the diluted enzyme was used in the assay.

The Kₘ for NH₄⁺ was estimated to be less than 0.2 mM. K⁺ at 10 mM instead of NH₄⁺ stimulated the activity about 2-fold. Stimulation by NH₄⁺ and K⁺ have been reported with the P-fructokinase from yeast (24) and sheep brain (25). Approximately 2-fold stimulation of enzymic activity was observed in the presence of 100 mM 2-mercaptoethanol when tested in the presence of NH₄⁺ (2 mM). Moreover, 300 mM 2-mercaptoethanol did not inhibit the reaction under those conditions.

Inhibition by ATP and Other Phosphorylated Compounds—
The specificity of various nucleoside triphosphates as inhibitors was examined and the results are shown in Fig. 3. It can be seen that UTP and ATP were effective inhibitors, whereas CTP was only moderately inhibitory even at 5 mM. ITP and GTP were not inhibitory at this concentration. However, 12 and 36 mM GTP inhibited 12 and 80%, respectively. Vinuela, Salas, and Sols (7) reported that yeast P-fructokinase was not inhibited by GTP at 2 mM. Inhibition by UTP of heart P-fructokinase was reported by Mansour (6).

The magnitude of the ATP inhibition was dependent on pH and the nature of the buffer, as shown in Fig. 4. At pH 9.0, no ATP inhibition was observed, but below pH 7.0 the inhibition increased considerably. At pH 7.0, the inhibition was much greater in imidazole buffer than in phosphate. Pyrophosphate at pH 7.0 inhibited the reaction completely. Mansour (6) also reported the lack of ATP inhibition at alkaline pH with heart P-fructokinase.

In the course of studies with ATP-regenerating systems, it

![Graph](http://www.jbc.org/content/240/12/4686/F2)

**Fig. 2.** Stimulation of P-fructokinase by NH₄⁺. The standard assay for P-fructokinase activity was used except that variable concentrations of NH₄Cl were added to the reaction mixture. For this experiment, the auxiliary enzymes were dialyzed overnight against 1.8 mM EDTA. P-fructokinase, which was kept in 2 M ammonium sulfate, was centrifuged and the supernatant solution was removed by decanting and by wiping the inner wall of the centrifuge tube with a piece of filter paper. The precipitate was then dissolved in a solution containing 50 mM Tris (pH 7.6), 1 mM EDTA, and 100 mM 2-mercaptoethanol. Immediately before use, P-fructokinase was diluted 1000-fold in the same solution, and 10 µl of the diluted enzyme was used in the assay.

![Graph](http://www.jbc.org/content/240/12/4686/F3)

**Fig. 3.** Inhibition of P-fructokinase by nucleoside triphosphates. The assay method A was used except that variable concentrations of UTP, ATP, ITP, or CTP were added to the reaction mixture. P-fructokinase (0.013 unit) was added to initiate the reaction.

![Graph](http://www.jbc.org/content/240/12/4686/F4)

**Fig. 4.** ATP inhibition of P-fructokinase as a function of pH and type of buffer. The assay conditions were the same as those in Fig. 3, except that 7.2 X 10⁻² M fructose-6-P, variable ATP concentrations, and the buffers (50 mM) were used as indicated. P-fructokinase (0.009 unit) was added to initiate the reaction. The numbers in parentheses indicate the pH of the buffer used.

### Table VI

**Inhibition of P-fructokinase by P-creatine and P-enolpyruvate**

Assay B was used except that 0.2 mM ATP, 0.9 mM glucose-6-P, P-glucose isomerase (5 units), and the indicated concentrations of P-creatine or P-enolpyruvate were present. P-fructokinase (0.008 unit) was added to start the reaction.

<table>
<thead>
<tr>
<th>Compound added</th>
<th>Concentration</th>
<th>ΔAbsorbance/min</th>
<th>Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>P-creatine</td>
<td>mM</td>
<td></td>
<td>%</td>
</tr>
<tr>
<td>0.0</td>
<td>0.054</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>1.1</td>
<td>0.043</td>
<td>20</td>
<td></td>
</tr>
<tr>
<td>2.7</td>
<td>0.030</td>
<td>44</td>
<td></td>
</tr>
<tr>
<td>5.5</td>
<td>0.015</td>
<td>72</td>
<td></td>
</tr>
<tr>
<td>P-enolpyruvate</td>
<td>mM</td>
<td></td>
<td>%</td>
</tr>
<tr>
<td>1</td>
<td>0.031</td>
<td>41</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>0.018</td>
<td>66</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>0.013</td>
<td>77</td>
<td></td>
</tr>
</tbody>
</table>
was noted that both P-enolpyruvate and P-creatine inhibited P-fructokinase activity (Table VI). This inhibition compli-

**Table VII**

**Reversal of P-fructokinase reaction and ATP inhibition**

The reaction mixture contained in 1 ml: 50 mM imidazole (pH 7), 0.3 mM TPN, 5 mM MgCl₂, fructose-1,6-di-P, ADP, ATP, as indicated, 0.35 unit of P-glucose isomerase, and 0.24 unit of glucose-6-P dehydrogenase. After hexose monophosphates, which were present as contaminants in fructose-1,6-di-P, were exhausted, the reaction was initiated by addition of 0.08 unit of P-fructokinase activity (Table VI). This inhibition could be counteracted by AMP, but not by IMP. NH₄⁺ and 2-mercaptoethanol, although stimulating the activity of P-fructokinase, did not affect the inhibition by ATP.

Numerous attempts were made to alter the degree of ATP inhibition. Among the treatments tested, heat treatment tended to increase the ATP inhibition. On the other hand, aging the enzyme as ammonium sulfate precipitate at 0°C for 2 weeks as well as chymotrypsin treatment, appeared to render the enzyme less sensitive to the ATP inhibition. These results could be interpreted on the basis that the preparation contains a mixture of ATP-sensitive and -insensitive forms of the enzyme (26) which have different stability characteristics.

**Discussion**

Hexokinase from brain has been partially purified in a particulate form by Crane and Sols (18), and a preliminary note on its solubilization and further purification has appeared recently (27). Purification of the ascites tumor enzyme was decided on because these cells contain approximately 40% of the hexokinase in a soluble form (16). In contrast to the yeast enzyme, the soluble ascites tumor enzyme is very sensitive to product inhibition by glucose-6-P, a property which was a prerequisite for the studies of control mechanisms in reconstructed systems of glycolysis which are reported in the accompanying paper. The reaction catalyzed by yeast hexokinase is readily reversible, whereas that catalyzed by ascites hexokinase is not. This discrepancy is difficult to explain, but may be associated with the fact that glucose-6-P is a potent inhibitor of the tumor enzyme.

The glucose-6-P inhibition of the ascites tumor enzyme was partly released by P₁, as was reported by Rose et al. (10) for red cell hexokinase. Glucose-6-P inhibition was found to be competitive with ATP, confirming the observations of Fromm and Zewe (20) with brain hexokinase. However, ADP was also found to be an inhibitor of the tumor hexokinase competitively with ATP.

The P-fructokinase preparation from rabbit muscle described in this paper has a specific activity of about 210. Previously, Ling, Byrne, and Lardy (28) reported a preparation with spe-

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sic activity of 130 from the same source, but the conditions of assay were different. The purified enzyme was markedly inhibited by excess ATP or UTP, but not by ITP. This latter observation is of particular interest for comparative studies in reconstructed systems because ITP serves also as a substrate for hexokinase and can be used as a substitute for ATP. Of particular significance is the reversal of the ATP inhibition of P-fructokinase by ADP, AMP, and P1 (4). Whereas the stimulatory effect of P1 is also shared by hexokinase, the latter enzyme is inhibited by ADP. This difference between P1 and ADP allows for a special role of Pi as a coordinated stimulator of glycolysis, as will be shown in the accompanying paper.

Another interesting property of P-fructokinase is the inhibition by P-enolpyruvate and P-creatine, intermediates far removed from the step catalyzed by P-fructokinase. The effect of P-enolpyruvate on this enzyme may be responsible for the inhibition of glycolysis in red blood cells by P-enolpyruvate and P-glycerate observed over 20 years ago by Dische (29), who with remarkable vision suggested that these intermediates may regulate glucose utilization by a type of feedback inhibition.

It is apparent from these findings and from reports in the literature that both hexokinase and P-fructokinase are readily influenced by numerous intracellular constituents. Determination of the concentration of a single component such as glucose-6-P gives, therefore, only limited information on the probable occurrence of a product inhibition because of the profound effects of P1, ATP, and other compounds on this phenomenon.

In view of these complexities, it was thought desirable to re-examine the reconstructed multienzyme system with tumor hexokinase under well controlled conditions. These studies are the subject of the accompanying paper (12).

SUMMARY

1. A soluble and stable hexokinase was purified from ascites tumor cells. In contrast to yeast hexokinase, this enzyme was very susceptible to product inhibition by glucose 6-phosphate and adenosine diphosphate. Both inhibitions were found to be competitive with ATP. The inhibition by glucose 6-phosphate was partly released by P1, but the ADP inhibition was not.

2. A highly purified and stable preparation of phosphofructokinase was isolated from rabbit muscle. The enzyme was stimulated by NH4+ and 2-mercaptoethanol and was highly susceptible to inhibition by ATP, UTP, phosphoenolpyruvate, and phosphocreatine. ITP served as a substrate but was not inhibitory up to 5 mm. The inhibition by ATP was dependent on pH and on the buffer used. The inhibition was released by several adenine nucleotides and by P1, but IMP, UMP, and GMP were inactive.

3. The significance of these findings for the evaluation of control mechanisms of carbohydrate metabolism is discussed.

REFERENCES
