Rabbit Brain Phosphofructokinase

COMPARISON OF REGULATORY PROPERTIES WITH THOSE OF OTHER PHOSPHOFRUCTOKINASE ISOZYMES*

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SUMMARY

A procedure was developed for the purification of phosphofructokinase from rabbit brain extracts. The enzyme was purified more than 100-fold with a yield of greater than 50%. It was found to have the same distribution of multiple molecular forms as found in the total brain extract, including one molecular species that is different from the more extensively studied muscle (heart) and liver (erythrocyte) isozymes.

The regulatory properties of brain phosphofructokinase were compared with those of muscle and liver phosphofructokinases at pH 7.1 and 7.4. All three enzymes were inhibited less at pH 7.4 than at 7.1. At pH 7.1 the sensitivity to ATP as an inhibitor decreased in the order liver > muscle > brain. At pH 7.4, the relative sensitivities of brain and muscle phosphofructokinases were reversed with the brain enzyme being inhibited at slightly lower concentrations of ATP. Other inhibitors acted synergistically with ATP. Sensitivity to inhibition by 3-phosphoglycerate and phosphoenolpyruvate decreased in the order muscle > brain > liver. With 2,3-diphosphoglycerate as an inhibitor, the sensitivity decreased in the order liver > muscle > brain. Muscle phosphofructokinase was the most sensitive to citrate inhibition. Creatine phosphate, a potent inhibitor of muscle phosphofructokinase, was completely ineffective as an inhibitor of liver and brain phosphofructokinase.

The actions of activators were evaluated at pH 7.4 in the presence of inhibitory concentrations of ATP. The three enzymes were almost equally responsive to the deinhibiting action of AMP and cyclic adenosine 3':5'-monophosphate, although the enzyme from liver required slightly higher concentrations. Muscle phosphofructokinase was the least sensitive to deinhibition by inorganic phosphate.

The results are discussed with respect to the varying modes of carbohydrate metabolism in the different tissues taking into consideration the tissue concentrations of the effectors and the variations of concentration in different physiological states. The enzymes from brain and liver are apparently chiefly controlled by the relative amounts of the adenine nucleotides and inorganic phosphate. An exception may be the erythrocyte, which has the same isozyme that is present in liver. Here, 2,3-diphosphoglycerate may play an important role in regulation. Muscle phosphofructokinase also responds to varying levels of adenine nucleotides but in vivo it is most likely that the principal regulatory effectors are creatine phosphate, citrate, and possibly, 3-phosphoglycerate.

Because of its established importance in the regulation of carbohydrate metabolism, phosphofructokinases from many sources have been studied extensively. The discovery (1, 2) of a new glycogen storage disease that results from a lack of this enzyme in muscle tissue and a subnormal amount of activity in the erythrocytes of these patients led to a number of studies on multiple molecular forms of this enzyme (3-12). Such investigations have concluded that not only is there more than one form of phosphofructokinase in mammalian tissues, but that the multiple forms have unique regulatory properties (7, 11).

In a study (12) of rabbit phosphofructokinase isozymes we have noted that skeletal muscle and heart have one molecular species, designated phosphofructokinase A, and liver and erythrocytes have a second, designated phosphofructokinase B. All other tissues have more complex distributions that include hybrids containing A and B monomers. In several tissues a third monomer type was observed, which was designated phosphofructokinase C. This molecular form, along with hybrids of C and A, is most abundant in brain tissue. Brain phosphofructokinase has been the subject of two previous studies. Lowry and Passonneau (13) reported a detailed kinetic analysis of sheep brain phosphofructokinase, and Kryzanowski and Matschinsky (14) compared the regulatory properties of a relatively crude preparation of sheep brain phosphofructokinase with those of a commercial preparation of rabbit skeletal muscle enzyme. In this latter report, however, it is impossible to distinguish those differences in enzymic properties that are species-specific from those that are organ-specific. Furthermore, in both studies, the yield of enzyme during the preparation was very low and it is possible that a single isozyme of several present in the brain may have been selectively purified in the procedure.

The present communication describes a procedure for the purification of brain phosphofructokinase with a relatively high yield and specific activity. The enzyme purified by this pro-

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procedure appears to have the same distribution of isozymes and hybrids as that observed in total brain extracts. The brain is highly dependent upon continuous energy production from glucose metabolism and hence the regulatory properties of such a key pacemaker as phosphofructokinase is of considerable interest. Presented here is a detailed comparison of the regulatory properties of the enzyme from rabbit brain with those of the enzymes from skeletal muscle and liver (isozymes A and B, respectively). The results are discussed in light of the different modes of carbohydrate metabolism of these tissues.

EXPERIMENTAL PROCEDURE

Materials—All nucleotides employed in these studies were obtained from P-L Biochemicals. Dithiothreitol, glycyglycine, tri-(hydroxyethyl)aminomethane (Tris), N-tris(hydroxymethyl)-methyl-2-aminoethanesulfonic acid (Tes), a-glycerol-1-P dehydrogenase, triosephosphate isomerase, and all phosphorylated sugar derivatives were obtained from Sigma Chemical Co. Phenazine methosulfate and nitro blue tetrazolium were products of Nutritional Biochemicals. Frozen rabbit liver and brain for the preparation of phosphofructokinase and aldolase were from young (8 to 12 weeks) New Zealand White rabbits of mixed sex and were purchased from Pel-Freez Biologicals, Rogers, Ark. Aldolase was prepared by the method of Taylor (15) and glyceroldehyde-3-P dehydrogenase by the method of Veltiek (16).

Rabbit muscle phosphofructokinase was prepared by a modified procedure of Ling et al. (17) and Parmeggiani et al. (18) according to the method of Kemp (7). The methods for the preparation of antisera against rabbit muscle phosphofructokinase and against rabbit liver phosphofructokinase have been described previously (12).

Assays.—Determination of enzymatic activity was performed with a Gilford model 2000 spectrophotometer at 260 nm and 8.2 pH. Activities were determined in a medium containing 25 mM glycylglycine, 25 mM glycerol-P, 1 mM EDTA, 6 mM MgCl2, 0.2 mM DPNH, 3 mM (NH4)2SO4, 0.1 mM dithiothreitol, 0.6 unit of aldolase, 0.3 unit of triosephosphate isomerase, 0.3 unit of glyceraldehyde-3-P dehydrogenase, and ATP, fructose-6-P, and other additions as indicated. One unit of enzyme is defined as that amount of enzyme that converts 1 mmole of fructose-6-P to fructose-1,6-P in 1 min in the assay system at pH 8.2 in the presence of 1 mM fructose-2,6-P and 1 mM ATP. For studies of the regulatory properties, the medium was buffered by 50 mM Tes titrated with KOH to the desired pH. KCl was then added to make the final concentration of potassium ions 150 mM. Other ingredients of the assay in a total volume of 3 ml and at the indicated pH included 1 mM EDTA, 0.2 mM DPNH, 1 mM dithiothreitol, 0.6 unit of aldolase, 0.3 unit of triosephosphate isomerase, 0.3 unit of glyceraldehyde-3-P dehydrogenase, and ATP and fructose-6-P at the indicated concentrations. MgCl2 was always present 5 mM in excess of the concentration of ATP. All enzymes and auxiliary enzymes were dialyzed extensively before use to remove ammonium sulfate. On the day of the experiment, a stock solution was made that contained all the reagents except ATP, phosphofructokinase, auxiliary enzymes, and fructose-6-P. These were added to the cuvette in the above listed order with a pause of 5 min prior to the addition of fructose-6-P to allow equilibration. In the studies of regulatory properties, 0.05 unit of enzyme was added in each assay. Potential inhibitors, where used, were always added after the addition of ATP. Rates were determined 4 to 6 min after starting the reaction by the addition of fructose-2,6-P. Protein determination was performed by the colorimetric method of Lowry et al. (20) with bovine serum albumin as a standard.

Other Procedures—Zone electrophoresis on cellulose acetate and the procedure for the quantitative precipitation of enzyme activities by antisera were carried out as described previously (12).

RESULTS AND DISCUSSION

Purification of Phosphofructokinase from Rabbit Brain

The procedure for the purification of brain phosphofructokinase has been successfully repeated many times and the following data are those from a typical preparation.

Step 1: Extraction—Two pounds of frozen brains were cut into small pieces with a meat cleaver and placed in a Waring Blendor containing 3 volumes of ice-cold 30 mM KF and 4 mM EDTA, all adjusted to pH 7.5 with KOH. The mixture was homogenized twice in a cold room for 45 s with a 30-s pause between blending periods. The homogenate was centrifuged for 20 min at 15,000 × g and 2°C in a Sorvall RC-2 centrifuge. The supernatant was decanted through glass wool, and the pH adjusted to 7.5 by the addition of 1 M Tris base.

Step 2: Ammonium Sulfate Precipitation—To the supernatant solution, solid ammonium sulfate was slowly added with stirring to a final saturation of 0.6. After the suspension was stirred for 1 hour, the precipitate was collected by centrifugation for 30 min at 15,000 × g and dissolved in 500 ml of a pH 8.0 solution containing 30 mM Tris, 30 mM ammonium sulfate, 2 mM EDTA, and 0.1 mM ATP. The solution was dialyzed against two changes of this buffer overnight. Solid ammonium sulfate was then added to a final saturation of 0.20. The suspension was stirred for 1 hour and then centrifuged at 15,000 × g for 20 min. The pellet was discarded and ammonium sulfate was added to bring the supernatant to a final saturation of 0.5. After stirring for 1 hour, the suspension was centrifuged at 15,000 × g for 20 min. The pellet was dissolved in 100 ml of the pH 8.0 buffer containing 30 mM Tris, 30 mM ammonium sulfate, 2 mM EDTA, and 0.1 mM ATP. This buffer will subsequently be referred to as column buffer. The solution was then dialyzed for 24 hours with two changes of this buffer. Insoluble material was removed by centrifugation at 15,000 × g. If considerable turbidity remained in the supernatant, it was removed by centrifugation at 90,000 × g for 1 hour.

Step 3: Fractionation on DEAE-Sepahex—A column with a 5-cm diameter was packed to a height of 40 cm with DEAE-Sepahex A-50 previously equilibrated with column buffer. The dialyzed fraction from Step 2 was applied to the column at a rate of 20 to 25 ml per hour. The column was then washed with the same buffer until the absorbance at 280 nm of the effluent dropped below 0.1 A unit. No activity was present in the initial effluent and wash. The enzyme was then eluted with a linear gradient of 0.03 to 0.4 mM ammonium sulfate in the same buffer described above (1500 ml in each gradient bottle) at a rate of 45 to 50 ml per hour, and fractions of 10 ml were collected. A typical elution profile is shown in Fig. 1. The presence of a second smaller peak on the descending part of the main peak was found in every preparation. The reason for this two-peak pattern in the ion exchange chromatographic procedure is not

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1 The abbreviations used are: Tes, N-tris(hydroxymethyl)-methyl-2-aminoethanesulfonic acid; cyclic AMP, cyclic adenosine 3':5'-monophosphate.
clear because electrophoretic analysis always showed the presence of multiple overlapping bands in both peaks of activity.

Those fractions with more than 1 unit per ml were pooled and the enzyme was precipitated by adding solid ammonium sulfate with constant stirring to 0.5 saturation. After 1-hour equilibration at 4°, the precipitate was collected by centrifugation for 30 min at 10,000 × g and redissolved in 30 ml of column buffer.

**Step 4: Heat Treatment**—The solution from the previous step was placed in a flask and heated in a water bath at 75–80°. The solution was stirred efficiently and the temperature was allowed to increase to 55°. The flask was then transferred to a constant temperature water bath and the temperature of the solution was maintained at 55° for 3 min. The suspension was then cooled to 10° or lower and centrifuged at 17,000 × g for 20 min. The sediment was discarded. The enzyme in the supernatant solution was precipitated by adding ammonium sulfate to a final saturation of 0.5 and the sediment obtained after centrifugation at 27,000 × g was dissolved in 5 ml of column buffer containing 1 mM dithiothreitol.

**Step 5: Gel filtration**—The enzyme solution from Step 4 was applied to a Bio-Gel A-1.5m column (3.0 × 80 cm) that had been equilibrated with column buffer plus 1 mM dithiothreitol. Elution was carried out with the same buffer. The rate of the flow was 8 to 20 ml per hour and 10-ml fractions were collected. The fractions containing more than 1 unit per ml were combined and precipitated by the addition of solid ammonium sulfate to 0.6 saturation. After the suspension had been stirred for 1 hour at 4°, the precipitate was collected by centrifugation at 27,000 × g for 30 min and redissolved in a small amount of column buffer containing 1 mM dithiothreitol.

**Properties of Brain Phosphofructokinase Preparation**

The results of the above described procedure are summarized in Table 1. The procedure produces a 55% yield of enzyme with a specific activity of about 40 units per mg. The enzyme is not homogeneous as indicated by the variation in specific activity across the peak of activity from the gel permeation chromatography. In comparison, the homogeneous muscle phosphofructokinase has a specific activity of 160.

At protein concentrations greater than 10 mg per ml, the enzyme was stable for several months in column buffer containing dithiothreitol, although it was sometimes necessary to dialyze the enzyme overnight against fresh buffer to restore maximal activity. At protein concentrations less than 0.1 mg per ml the enzyme was much less stable and the presence of 0.1 mM ATP and dithiothreitol were necessary for the maintenance of activity for periods of several hours.

Because all fractions containing significant amounts of activity in the ion exchange elution were pooled for this procedure (Fig. 1), the preparation contains all of the isozyme hybrids that are present in the crude extract (12) and no component was selectively purified by the procedure. This was confirmed by a comparison of the purified enzyme and the crude brain extract by means of cellulose acetate electrophoresis and of the relative amount of activity precipitated by antisera prepared against phosphofructokinase A and phosphofructokinase B (12). From the relative intensities of the activity bands observed in the electrophoretic analysis, it is estimated that both the crude extract and the purified preparation contain about 60 to 70% C-type subunits and 30 to 40% A-type subunits.

Analysis of individual fractions of the elution by means of cellulose acetate electrophoresis showed that components present in the descending part of elution peak migrated toward the anode more rapidly than those present in the earlier fractions. Altogether five electrophoretic species, presumably isoforms A and C and their hybrids (12), could be detected, but only three bands could be clearly distinguished when either the crude extract or the semipurified enzyme after Step 5 of the purification procedure was analyzed by electrophoresis on cellulose acetate strips. This is due to the fact that both the A- and A-C isoforms are present in small amounts relative to the other electrophoretic species.

It was shown previously (12) that C-type isozyme in brain can be separated from other phosphofructokinase components by treating an extract of brain tissues with excess anti-A-type phosphofructokinase serum or with a combination of anti-A-type and anti-B-type phosphofructokinase sera. This technique was not practical for preparing the amount of enzyme required for an extensive kinetic study. Attempts to isolate this isozyme by rechromatography of the purified brain enzyme on DEAE-Sephadex under various conditions did not achieve resolution of the individual components. Preliminary attempts at iso-electric focusing led to loss of activity.

In all of the kinetic studies to follow, the purified enzyme obtained from Step 5 was compared with homogeneous preparations of skeletal muscle enzyme (isoform A) and highly purified preparations from liver (isoform B). In some instances (not shown), the kinetic regulatory properties of phosphofructokinase from crude extracts of brain or from various stages of the purification were compared with that from Step 5 and no detectable differences were noted. Similarly, a preparation of muscle phosphofructokinase that was purified by a procedure nearly identical to that of Ling et al. (17), which avoided both the heat treatment and the crystallization steps, was found to have properties identical to those reported here. Assuming that, as suggested for a number of other enzymes (21), the various molecular forms of phosphofructokinase are the products of different genes in the same cell, then the comparison of the regulatory properties of the brain phosphofructokinase with those of isoform A, the only form of phosphofructokinase found in rabbit skeletal and cardiac muscle, and those of isoform B, the predominant isoform species in liver and erythrocytes (12), would therefore provide a valid comparison of the enzymes that are present in each cell of these different tissues. Considering the key role of phosphofructokinase in the regulation of carbohydrate metabolism, any differences should be very significant to the varying modes of metabolism of these tissues.

**Table I**

<table>
<thead>
<tr>
<th>Step</th>
<th>Volume</th>
<th>Concentration</th>
<th>Total units</th>
<th>Protein</th>
<th>Specific activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Extraction</td>
<td>2300</td>
<td>3.2</td>
<td>7400</td>
<td>8.7</td>
<td>0.37</td>
</tr>
<tr>
<td>2. Ammonium sulfate</td>
<td>123</td>
<td>52</td>
<td>6400</td>
<td>29</td>
<td>1.8</td>
</tr>
<tr>
<td>3. DEAE-Sephadex</td>
<td>41</td>
<td>125</td>
<td>5100</td>
<td>23</td>
<td>5.4</td>
</tr>
<tr>
<td>4. Heated supernatant</td>
<td>40</td>
<td>125</td>
<td>5000</td>
<td>8.9</td>
<td>14</td>
</tr>
<tr>
<td>5. Bio-Gel A-1.5m</td>
<td>10</td>
<td>410</td>
<td>4100</td>
<td>10.3</td>
<td>40</td>
</tr>
</tbody>
</table>

**Comparison of Regulatory Properties of Purified Enzymes from Brain, Muscle, and Liver**

**ATP Inhibition**—The activity of phosphofructokinase is known to be sensitive to inhibition by one of its substrates, ATP, and to be further regulated by a large variety of metabolites including inorganic phosphate, citrate, creatine phosphate,
phosphoenolpyruvate, adenine nucleotides, and several glycolytic intermediates (see Refs. 22 and 23 for review). The sensitivity of isozymes A and B to ATP and the other effectors has been shown to be different (7), and one might well predict that brain phosphofructokinase may also be under unique regulatory control. In the earlier study from this laboratory (7), a detailed comparison of kinetic regulatory properties of muscle and liver phosphofructokinase was carried out using a buffer system containing glycylglycine and glycerophosphate. Unfortunately, this buffer is not ideal because these two compounds are themselves weak effectors. Consequently, a more inert buffer, Tes, was chosen for the present study. Unlike their behavior in the glycylglycine and glycerophosphate buffer, phosphofructokinases from all three sources, brain, liver, and muscle, are extremely unstable in Tes solutions. The addition of 1 mM dithiothreitol and 0.1 mM ATP, however, stabilized the three enzymes. The assay medium contained 50 mM Tes, 150 mM KCl, 1 mM EDTA, 1 mM dithiothreitol, auxiliary enzymes, and MgCl\textsubscript{2} present at a concentration that was 5 mM in excess of the concentration of ATP.

Fig. 2A shows the effect of increasing ATP concentrations on the activity of each of the three enzymes at pH 7.1. The muscle and liver enzymes were much more sensitive to ATP inhibition in the Tes buffer system than was observed previously in glycylglycine-glycerophosphate buffer (7). With the low fructose-6-P concentration employed to obtain the data in Fig. 2A (0.4 mM fructose-6-P) the liver enzyme (isozyme B) was almost completely inactive even at ATP concentrations lower than 0.1 mM. Liver phosphofructokinase activity was measurable only in the presence of high concentrations of fructose-6-P (4 mM) or in the presence of other activators. In the presence of 4 mM fructose-6-P, the concentration of ATP required to achieve half maximal inhibition of the liver enzyme was 0.4 mM. In contrast, the brain enzyme required about 1.2 mM ATP for half-maximal inhibition in the presence of 4 mM fructose-6-P. At any given concentration of fructose-6-P, the extents of inhibition of the enzymes were in the order liver > muscle > brain. With the pH of the assay mixture at 7.4 (Fig. 2B), all three enzymes were much less sensitive to inhibition by ATP.

The decreased sensitivity of isozyme A (heart, muscle) to ATP at alkaline pH is well described in the literature (22, 23). The concentration of ATP required to inhibit liver phosphofructokinase by 50% is very low (0.7 mM) as compared to the concentrations of ATP required to inhibit the muscle or the brain enzyme to the same extent. The relationship between the muscle enzyme and the brain enzyme with respect to their sensitivity to ATP inhibition is reversed at the higher pH. At pH 7.4, brain phosphofructokinase was inhibited to a greater extent than the muscle enzyme at any inhibitory level of ATP. At pH 7.4, the potency of inhibition by ATP decreases in the order phosphofructokinase A (muscle, heart), brain phosphofructokinase, and phosphofructokinase B (liver, erythrocytes). The reported tissue levels of ATP decrease in the same order; ATP in skeletal muscle and heart is 5 mM or higher (24, 25), in mouse brain ATP is about 2.5 mM (26), and in liver and erythrocytes, the reported values are less than 2 mM (27, 28).

All three enzyme preparations display decreased sensitivity to ATP inhibition at more alkaline pH, but the enzyme from muscle (isozyme A) appears to be the most strongly affected as shown by a comparison of the data at pH 7.1 and 7.4. It is possible that the high sensitivity of isozyme A to pH may be a specific adaptive mechanism for contractile tissues. Trivedi and Danforth (29) first noted the high sensitivity of muscle phosphofructokinase to pH and suggested that shifts in intracellular pH, such as those that may accompany creatine phosphate hydrolysis, are important to the regulation of phosphofructokinase in vivo. Muscle undoubtedly displays more dramatic intracellular shifts in pH due to the large variations in glycolytic flux and in accompanying lactic acid levels.

Other Inhibitors—It is difficult to readily assess the relative potency of other inhibitors with respect to the isozymes because of the differing sensitivity of the isozymes to ATP inhibition and because ATP acts synergistically with the other inhibitors (30); that is, an increase in ATP concentration brings about an even greater sensitivity to the inhibitor. As a result it was usually necessary to compare data obtained with several different concentrations of ATP. Furthermore, because the relative ATP inhibition changes with pH, the effects of inhibitors were evaluated at pH 7.1 and pH 7.4.

The effects of citrate, phosphoenolpyruvate, and phosphoglycerates on the activities of muscle and brain phosphofructokinase at pH 7.1 are shown in Fig. 3. In the presence of 0.2 mM ATP and 0.4 mM fructose-6-P, higher concentrations of inhibitors were required in each case for inhibition of the brain enzyme to the same degree as the muscle enzyme. The liver enzyme was not studied under these conditions because it is already completely inhibited by 0.2 mM ATP. Data from Fig. 3 along with data obtained under several other conditions are given in Table II. Of those inhibitors shown in Fig. 3, citrate and 3-phosphoglycerate are probably the most significant with regard to muscle phosphofructokinase. The muscle enzyme is very sensitive to citrate inhibition and previous studies have shown that the enzyme from liver is much less potently inhibited. Both muscle and brain enzymes are more sensitive to 3-phosphoglycerate than they are to phosphoenolpyruvate and analyses of substrate levels in heart (25) and brain (26) have shown that 3-phosphoglycerate levels exceed phosphoenolpyruvate levels by 4- to 10-fold.

Fig. 4 and Table II show the inhibitory effect of phosphoglycerates on all three enzymes at pH 7.4. The effect of phosphoenolpyruvate was also examined under the conditions of Fig. 4 (0.4 mM ATP-1.0 mM fructose-6-P) but no inhibition of

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**Diagram**

Fig. 2. ATP inhibition of phosphofructokinases. $V_t$ is the velocity observed at pH 8.2 in the glycylglycine-glycerophosphate buffer described under “Experimental Procedure.” Data were obtained from assays at pH 7.1 (A) and pH 7.4 (B) in the Tes buffer system described in the text. Fructose-6-P was present at 0.4 mM and at 1 mM in B. ○, brain phosphofructokinase; □, muscle phosphofructokinase; △, liver phosphofructokinase.
Inhibitor (mM)

**Table II**

Inhibition of phosphofructokinase isozymes by glycolytic intermediates

<table>
<thead>
<tr>
<th>Reaction conditions</th>
<th>Concentration of phosphofructokinase isozymes required to achieve 50% inhibition</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Inhibitor</td>
</tr>
<tr>
<td>3-Phosphoglyceric acid...</td>
<td>7.1</td>
</tr>
<tr>
<td>Phosphoenolpyruvate...</td>
<td>7.1</td>
</tr>
<tr>
<td>2,3-P₂-glyceric acid...</td>
<td>7.1</td>
</tr>
<tr>
<td>3-Phosphoglyceric acid...</td>
<td>7.4</td>
</tr>
<tr>
<td>Phosphoenolpyruvate...</td>
<td>7.4</td>
</tr>
<tr>
<td>2,3-P₂-glyceric acid...</td>
<td>7.4</td>
</tr>
<tr>
<td>2,3-P₂-glyceric acid...</td>
<td>7.4</td>
</tr>
</tbody>
</table>

Liver or brain enzyme and only 10% inhibition of muscle enzyme was detected at 8 mM phosphoenolpyruvate. 3-P-glycerate was found to inhibit the muscle enzyme at lower concentrations than the brain enzyme, whereas liver phosphofructokinase was only slightly inhibited even at a relatively high concentration of this substance. 2,3-Diphosphoglycerate is, as shown previously (7), a good inhibitor of phosphofructokinase from liver. This intermediate is only a weak inhibitor of the brain and muscle enzymes, and inhibition is only significant when ATP levels are increased to inhibitory concentrations (Table II). The muscle enzyme is the more sensitive of the two. Liver phosphofructokinase or isozyme B is also the major isozyme species of rabbit erythrocytes, and Tarui et al. (31) have purified this isozyme from rabbit erythrocytes and also have described the inhibition of the enzyme by 2,3-diphosphoglycerate. Only in erythrocytes does one find 2,3-diphosphoglycerate concentrations high enough to play a significant role in the regulation of phosphofructokinase. A Pasteur effect, the inhibition of glucose utilization by oxygen, has been described for erythrocytes (32). Because it is also known that diphosphoglycerate is preferentially bound by deoxyhemoglobin (33), it is possible that the Pasteur effect of erythrocytes is due to the release of sequestered diphosphoglycerate during the oxygenation of hemoglobin, making it available for the inhibition of erythrocyte phosphofructokinase.

Creatine phosphate is present in the brain as well as muscle and heart, and for this reason it was thought to be a potential inhibitor of brain phosphofructokinase. However, the concentration in brain has been reported to be about 2.5 mM (26), which is considerably lower than the 20 to 30 mM found in heart and muscle. In any case, the brain enzyme is very insensitive to the presence of creatine phosphate as shown in Fig. 5. At pH 7.1 and with the other conditions described in the figure, the concentration of creatine phosphate required to give 50% inhibition of muscle phosphofructokinase was about 1.9 mM. In contrast, no inhibition of the brain enzyme was observed at a creatine phosphate concentration of 16 mM even when the

![Figure 3](https://example.com/image3.png)

**Figure 3.** Effects of inhibitors at pH 7.1. Vₜ is defined in Fig. 2. Fructose-6-P was present at 0.4 mM and ATP at 0.2 mM except as indicated in the following: ♂, brain phosphofructokinase; ●, brain phosphofructokinase with 0.65 mM ATP; □, muscle phosphofructokinase.

![Figure 4](https://example.com/image4.png)

**Figure 4.** Inhibition by phosphoglycerates at pH 7.4. Vₜ is defined in Fig. 2. Fructose-6-P was present at 1 mM and ATP at 0.4 mM. ♂, brain phosphofructokinase; □, muscle phosphofructokinase; Δ, liver phosphofructokinase.

![Figure 5](https://example.com/image5.png)

**Figure 5.** Inhibition of phosphofructokinases by creatine phosphate. Conditions of assay given in figure with ATP concentrations given next to the appropriate curve. ♂ and ●, brain phosphofructokinase; □, muscle phosphofructokinase; Δ, liver phosphofructokinase.
concentration of ATP was increased to inhibitory levels. At pH 7.4, the muscle enzyme is less sensitive to creatine phosphate but is still inhibited by a concentration within the physiological range. Under these conditions also the brain enzyme is not inhibited. As noted previously (7), phosphofructokinase B is also not inhibited by creatine phosphate.

**Activators**—Inorganic phosphate and the mono- and diphosphoadenine nucleosides are potent activators of mammalian phosphofructokinases. Their effects are readily observed in the presence of inhibitory concentrations of ATP. Because of the differential sensitivities of the multiple molecular forms of phosphofructokinase to the inhibitory effect of ATP, the best comparison of the relative effects of the activators would be carried out at a point where the isozymes are inhibited to the same extent. Because the slope of the inhibition curve is fairly steep, this is not easily done. The most convenient concentrations were those which inhibited each by 95%, at pH 7.4 with 1 mM fructose-6-P; these concentrations were 2 mM for the liver enzyme, 8.5 mM for the brain, and 9.5 mM for muscle phosphofructokinase (see Fig. 2).

Fig. 6 shows the activating action of AMP and cyclic AMP on the three phosphofructokinase preparations. In agreement with previous studies, cyclic AMP is effective at lower concentrations than AMP (7), and the liver enzyme is slightly less sensitive than muscle phosphofructokinase to both activators. The differences among all three enzymes are very small. The brain enzyme is at least as sensitive to the activators as the muscle enzyme although the muscle enzyme reaches the highest activity of the three. The brain enzyme under these conditions has a $K_a$ for AMP of about 0.1 mM and $K_a$ for cyclic AMP of approximately 0.05 mM.

The effect of inorganic phosphate under the same conditions as that of Fig. 6 are shown in Fig. 7. A clear difference is observed in the responses of the three enzymes to increasing concentrations of inorganic phosphate. The phosphofructokinases of brain and liver show almost identical sensitivities with $K_p$S of about 1 mM, whereas the enzyme of muscle has $K_p$ for phosphate of about 3.5 to 4.0 mM.

Some Conclusions—Because of the complex interactions of pH, substrates, products, and effectors with phosphofructokinase, it is difficult to make direct extrapolations of effects seen at a given concentration in vitro to the in vivo situation. For example, over quite a range of concentrations, the inhibitory effect of one substrate, ATP, varies inversely with the concentration of the other substrate, fructose-6-P. Furthermore, the enzyme is increasingly sensitive to the actions of inhibitors such as phosphoglycerates and creatine phosphate as the concentration of ATP is increased. In the studies reported here, we have generally tried to isolate phenomena; that is, to change one variable at a time. In vivo, however, the enzyme is exposed to a complex medium containing, to some extent, all of the inhibitors and activators that have been mentioned. To make any sense of the situation, one has to consider what metabolites are at concentrations that can cause effects on a given isozyme, and then whether the concentration of the metabolite fluctuates over a range that can influence the enzyme. Muscle phosphofructokinase has a number of likely regulators. In general, the muscle enzyme appears to be the most sensitive to effectors of the three enzymes. Of all tissues, muscle is most subject to striking changes in the rate of glycolytic flux under normal conditions. At rest, muscle uses very little or no glucose or glycogen, whereas very high rates of glycolysis occur during contraction and recovery. Citrate, which is at high levels when fatty acids are being metabolized in muscle and heart, is a potent inhibitor and may well explain the inhibition of glycolysis by fatty acid utilization. Creatine phosphate is an even more important regulator because it is the first energy reservoir to be exhausted in stressed muscle (34). The large excess of creatine phosphate over ATP concentrations and the equilibrium catalyzed by creatine kinase assures a high ratio of ATP to ADP and AMP under most conditions. Hemberg et al. (35) have shown that the levels of ATP do not vary greatly under conditions where contractions induced by electrical stimulation have caused a profound increase in glycolytic flux. This increase in flux could be the result of falling creatine phosphate levels which would lead to dephosphorylation of phosphofructokinase, pyruvate kinase (36), and glycoaldehyde-3-P dehydrogenase (37). Regulation of phosphofructokinase by changing levels of ATP, ADP, and AMP may only be significant under conditions of dire stress where energy reserves are severely depressed.

As shown previously (9), phosphofructokinase B is comparatively insensitive to inhibitors at physiologically significant concentrations with the exception of ATP and 2,3-diphosphoglycerate. Brain phosphofructokinase is also less sensitive to the effectors with the exceptions of inhibition by ATP, and activation by adenine nucleotides and inorganic phosphate. Because the brain is not subject to the tremendous differences in energy demands that are observed in muscle, it maintains a fairly constant rate of glucose metabolism. It does respond to ischemia by an increased rate of glucose metabolism (26) and...
this response could be due entirely to changes in adenine nucleotides and inorganic phosphate. It should be noted that the brain is considered to be rather intolerant to anoxia.

In view of the fact that as much as one-half of the total brain phosphofructokinase is phosphofructokinase A (muscle), most of which is present as hybrids with C-type, it is surprising that the brain preparation is so insensitive to inhibitors of A-type phosphofructokinase. This is particularly striking in the case of creatine phosphate, a fairly potent inhibitor of phosphofructokinase A that has no effect on the brain enzyme. Because we felt that the brain enzyme may have been desensitized during the preparation, we examined the effect of creatine phosphate on the enzyme at various stages of purification but found no differences in the regulatory properties. It is also unlikely that the brain enzyme has been desensitized because it does show greater sensitivity to several effectors than does phosphofructokinase A; namely, ATP at pH 7.4 and inorganic phosphate.

One explanation for the insensitivity to creatine phosphate is that the phosphofructokinase C does not bind creatine phosphate and that hybrids that contain monomers of C are incapable of undergoing the allosteric transition to an inhibited state in the presence of creatine phosphate. In other words, the A monomers cannot operate independently. If this is the correct explanation, then hybrids of isozymes A and B may also have properties that are not simply an average of the properties of the two isozymes. This would be significant for several tissues, including adipose tissue, wherein most of the phosphofructokinase is in the form of A and B hybrids (10, 12).

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Rabbit Brain Phosphofructokinase: COMPARISON OF REGULATORY PROPERTIES WITH THOSE OF OTHER PHOSPHOFRACTOKINASE ISOZYMES

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