Regulation of Fructose-2,6-bisphosphate Content in Rat Hepatocytes, Perfused Hearts, and Perfused Hindlimbs*

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A method has been developed to specifically measure fructose-2,6-bisphosphate in tissues. After elimination of the hexose-6-phosphate present in the extract, fructose-2,6-bisphosphate is hydrolyzed and the acid-revealed fructose-6-phosphate is measured in a coupled enzymatic assay with bacterial NADH-linked luciferase. Fructose-2,6-bisphosphate was found in all tissues studied; and, in order of increasing concentration, epipidymal fat, kidney, skeletal muscle, lung, heart, liver, and brain contain 0.1 to 2.3 nmol/g of tissue. Values lower than 0.1 nmol/g cannot be accurately measured and represent the limit of detection of the method.

Incubation of isolated hepatocytes from fed rats in the presence of 20 mM alanine, pyruvate, glycerol, lactose, lactate, tagatose, fructose, and glyceraldehyde caused, in order of increasing effect, a 2- to 10-fold decrease in fructose-2,6-bisphosphate content. Incubation in the presence of 20 mM dihydroxyacetone, galactose, mannose, xylitol, arabinose, or ribose had little or no effect on the concentration of fructose-2,6-bisphosphate. When hepatocytes were incubated under anoxic conditions or in the presence of carbonyl cyanide m-chlorophenylhydrazone, a mitochondrial uncoupler, the fructose-2,6-bisphosphate content was decreased by 40 and 60%, respectively.

In perfused rat muscle, insulin and epinephrine, respectively, caused a 2- and 4-fold increase in fructose-2,6-bisphosphate and a stimulation of glycolysis. Electrical stimulation caused muscular contraction and a 2.5-fold increase in lactate production, but decreased fructose-2,6-bisphosphate. It also abolished the increase in this metabolite induced by epinephrine.

In perfused rat hearts, epinephrine caused the activation of protein kinase and phosphorylase but did not alter the fructose-2,6-bisphosphate content.

Fructose-2,6-bisphosphate is a very potent stimulator of liver and muscle phosphofructokinase (1, 2), and changes in its concentration in the liver have been related to changes in glycolytic fluxes in this tissue (3–5). In hepatocytes, glucose, vasopressin, phenylephrine, and the ionophore A23187 increase its concentration, whereas glucagon decreases it (6–8).

The existing method of measuring fructose-2,6-P₂ is based on its ability to stimulate phosphofructokinase (4–8). Since tissue extracts contain other ligands which alter phosphofructokinase activity, the concentration of which can vary under the conditions studied, this "stimulation assay" of fructose-2,6-P₂ is not entirely specific and another method has been developed. Advantage was taken of the remarkable acid labil-ity of fructose-2,6-P₂ (2, 3). After elimination of the endogenous hexose-6-P, the acid-revealed fructose-6-P was measured in a coupled enzymatic assay with bacterial NADH-linked luciferase (9).

This method was applied to study the effects of various sugars, gluconeogenic precursors, and anoxia on the content of fructose-2,6-P₂ in isolated hepatocytes. It was also applied to various rat tissues and more particularly to skeletal muscle and heart. Activation of muscle phosphofructokinase following epinephrine treatment has been reported (10, 11) and purified muscle phosphofructokinase can be phosphorylated by the cyclic AMP-dependent protein kinase (12). However, phosphorylation does not apparently result in a change in the activity of the enzyme (12). Fructose-2,6-P₂, which is a potent stimulator of the muscle enzyme (1, 2), therefore appears as a potential regulator. Changes in its concentration might explain the effect of epinephrine on phosphofructokinase and hence on glycolysis. Thus, the effect of epinephrine, insulin, and electrical stimulation on fructose-2,6-P₂ content was studied in perfused rat hindlimbs, and the effect of epinephrine was studied in perfused rat hearts.

**EXPERIMENTAL PROCEDURES**

**Preparation of Tissue Samples—**Methods for the preparation and incubation of hepatocytes have been described previously (13). After 20 min of incubation at 37 °C, the various agents or substrates were added to the cell suspension and the incubation was continued for 15 min. The cells (about 100 mg) were then collected by rapid centrifugation (30 s, Damon IEC, HN-S centrifuge) and the cell pellets were frozen in liquid nitrogen and kept at −70 °C until further processing. The pellet was extracted in 1.5 ml of extraction buffer and further treated as described below.

**Rat hindlimbs were perfused as described previously (14) at 37 °C with Krebs-Henseleit bicarbonate buffer (15) containing 10 mM glucose, 4% (w/v) bovine serum albumin, and 30% (v/v) washed aged human erythrocytes. The gas phase was CO₂/O₂ (59:41). After 20 min of perfusion, hormones were added by continuous infusion and the perfusion continued for 30 min. Muscle contraction was produced by electrical stimulation (5 volts, 10 ms intervals, 10 pulses/s; Grass S9 stimulator). The electrodes were applied on the posterior wall of the abdomen and maintained for 2 min at the end of the perfusion. The thigh muscles were freeze-clamped (16) and pulverized in a percussion mortar at the temperature of liquid N₂.

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§Supported in part by National Institutes of Health Grant AM 18699. Investigator, Howard Hughes Medical Institute.

1 The abbreviations used are: fructose-2,6-P₂: fructose-2,6-bisphosphate; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.
Rat hearts were perfused as described elsewhere (17) at 37 °C with Krebs-Henseleit bicarbonate buffer (15) containing 5 mM glucose. The gas phase was CO\(_2\):O\(_2\) (5:95). After 15 min of perfusion, epinephrine (5 \(\mu\)M, final concentration) was added to the perfusion medium and the perfusion continued for 2 or 5 min. Hearts were freeze-clamped and powdered as described above.

Measurement of Fructose-2,6-P~ The method consists of the measurement of fructose-6-P formed by acid hydrolysis of fructose-2,6-P~. The hexose-6-P present in the tissue extracts is first removed by an enzymatic step, followed by a purification on Dowex AG1-X8. The gas phase was CO\(_2\):O\(_2\) (5:95). After 15 min of perfusion, epinephrine (5 \(\mu\)M, final concentration) was added to the perfusion medium and the perfusion continued for 2 or 5 min. Hearts were freeze-clamped and powdered as described above.

Preparation of Extracts—The extreme acid lability of fructose-2,6-P~ prevents the use of acid for the deproteinization of tissues. Heat treatment was therefore used and the frozen powdered tissues (about 500 mg) were homogenized in 2.5 ml of hot (80 °C) medium using a motor driven Teflon pestle. Homogenates were then kept for 10 min at that temperature. As shown in Fig. 1, the recovery of added fructose-2,6-P~ was influenced by the pH of the medium. Routinely the extraction was performed in a medium (50 mM KF, 2 mM ethylene glycol bis(\(\beta\)-aminoethyl ether)-N,N,N',N'-tetraacetic acid, 10 mM Hepes, pH 7.5, 25 mM pyruvate, 10 \(\mu\)g of both phosphoglucose isomerase and glucose-6-phosphate dehydrogenase, and 5 \(\mu\)g of lactate dehydrogenase were added. After 30 min of incubation at 25 °C, 0.2 ml of the same solution was again added and the incubation continued for another 30 min. The reaction was stopped by heating for 5 min at 80 °C. After 60 min at 25 °C, 99.8 ± 0.08% (n = 6) of 0.5 \(\mu\)M of fructose-6-P was eluted. Fifty mg of charcoal (0.3 ml of a suspension containing 1 part of charcoal by weight for 5 parts of water) was added. The slurry mixture was centrifuged (5 min, Eppendorf microfuge) and 1.5 ml of the supernatant was diluted with the same volume of water and applied to a column (0.7 cm x 3 cm) of Dowex AG 1-X8 (chloride form). The column was successively washed with 3 ml of water, 3 ml of 0.15 M NaCl, and 1.5 ml of 0.2 M NaCl. Fructose-6-P was eluted with 1.5 ml of 0.4 M NaCl and 1.5 ml of 0.5 M NaCl (Fig. 2). In this fraction, fructose-6-P was almost undetectable (less than 0.01% of the starting material) and recovery of fructose-2,6-P~ by these steps was 95.9 ± 0.3% (n = 4).

Charcoal treatment was found to be very useful in removing some material which inhibited the last step of the assay, namely the measurement of NADH in the luciferase coupled reaction. The addition of a heat-treated extract of liver cells (corresponding to 5 pg of liver/test) caused a 90.2 ± 3.5% (S.E. for 6 observations) inhibition of the luciferase assay. This inhibition was only 28.7 ± 1% (S.E. for 6 observations) after charcoal treatment.

Hydrolysis of Fructose-2,6-P~ The specificity of the method is based on the unique acid-lability of fructose-2,6-P~, Glucose-1,6-P~ and fructose-1,6-P~ which may give rise to hexose-6-P by acid hydrolysis, are much less acid-sensitive than fructose-2,6-P~. In agreement with previous reports (2, 3), we found that after 10 min at pH 2 at room temperature, more than 95% of fructose-2,6-P~ was hydrolyzed, whereas less than 1% of glucose-1,6-P~ or fructose-1,6-P~ was destroyed. Hydrolysis was performed as follows.

Step 1. Tissue extract
PH 8, 80 °C for 10 min.
Step 2. Oxidation of fructose-6-P and glucose-6-P into 6-phosphogluconate in the presence of NAD, an excess of pyruvate, phosphoglucose isomerase, glucose-6-P dehydrogenase, and lactate dehydrogenase.
60 min at 25 °C.
Step 3. Purification
Charcoal treatment and chromatography on Dowex AG 1-X8 (chloride form).
Step 4. Hydrolysis of fructose-2,6-P~
PH 2, 25 °C for 10 min.
Step 5. Measurement of fructose-6-P
1. Formation of NADH from NAD in the presence of phosphoglucose isomerase and glucose-6-phosphate dehydrogenase.
2. Measurement of NADH by a bioluminiscence system containing a bacterial NADH-linked luciferase.

**Schematic 1**

**Procedure for the measurement of fructose-2,6-P~**

**FIG. 1. Recovery of fructose-2,6-P~ after 10 min at 80 °C at various pHs.** The extraction medium containing 50 mM KF, 2 mM ethylene glycol bis(\(\beta\)-aminoethyl ether)-N,N,N',N'-tetraacetic acid and 10 mM Hepes was brought to the indicated pH at 25 °C. The medium was then heated to 80 °C. 5 nmol of fructose-2,6-P~ were added, and fructose-2,6-P~ remaining after 10 min at 80 °C was measured. Because of the shift of the dissociation constant with temperature, pH 9.25 at 25 °C corresponds to pH 8 at 80 °C.

**FIG. 2. Elution profile of fructose-6-P and fructose-2,6-P~ from a column of Dowex AG1-X8 (chloride form)**. 5 nmol of fructose-2,6-P~ was applied. A stepwise gradient of NaCl was applied as indicated by the arrows, and fractions of 1.5 ml were collected. The elution profile of fructose-6-P~ corresponds to a sample which contained 0.5 nmol of fructose-6-P~ and which had been first treated as described in the text with NAD, pyruvate, glucose-6-P dehydrogenase, phosphoglucone isomerase, and lactate dehydrogenase to remove the majority of fructose-6-P~. The reaction was stopped by heating for 5 min at 80 °C and the mixture was applied to a column of Dowex AG1-X8. The remaining, unreacted fructose-6-P~ was eluted with the stepwise gradient of NaCl.
A 1-ml sample of the effluent was treated with 0.1 ml of 0.6 M HClO₄ for 10 min at room temperature and then neutralized with a mixture of 3 M KOH and 3 M KHC₂O₄. Another 1-ml sample served as a control and was treated first with the base, then with the acid. The two samples were left for 60 min at 0 °C to allow the precipitation of KCIO₃. The measurement of fructose-6-P was then performed on samples of both the acid-treated and the control effluents. Samples of 0.3 ml were incubated for 30 min at room temperature in a total volume of 0.6 ml in the presence of 50 nmol of NAD, 10 pmol of Hepes, pH 7.5, and 10 μg of both phosphoglucone isomerase and glucose-6-phosphate dehydrogenase. A blank was run without enzymes and an internal standard was obtained by adding 25 pmol of fructose-6-P to another sample. The reaction was complete after 20–30 min and aliquots of 0.2 ml were taken to measure in duplicate the NADH formed by the luciferase method. The difference between the acid-treated effluent and the control corresponds to fructose-2,6-P₂.

**Measurement of NADH by Bacterial NADH-linked Luciferase**

The principle of the method as well as the experimental details are described elsewhere (9). The assay was performed as follows: 0.2 ml of a solution of FMN (5 μg/ml of 50 mM potassium phosphate buffer, pH 7.0, containing 0.5 mM EDTA), 0.010 ml of decyl aldehyde (5 μl in 15 ml of methanol), and 0.05 ml of luciferase (0.5 mg of enzyme/ml of 50 mM potassium phosphate buffer, pH 7.0, containing 0.5 mM EDTA, 10% glycerol, 1 mM dithiothreitol, and 0.1% (w/v) defatted bovine serum albumin) were successively added to 0.2-ml samples containing up to 10 pmol of NADH. The mixture was vigorously vortex-mixed and the light intensity was measured in a spectrophotometer equipped with an integrator-timer (Amino Chem-Glow photometer and integrator). As recommended (9), the second 30-s readings were the values used. This assay is sensitive to salts, as illustrated in Fig. 3. Since the fractions containing fructose-2,6-P₂ were eluted with 0.4 M 340 nm, and solutions of fructose-6-P were titrated by enzymatic reactions of NADH were standardized by measuring the absorbance at 340 nm, and solutions of fructose-6-P were titrated by enzymatic method (20). Phosphorylase (22) and protein kinase activity ratio (23) were measured as previously described.

**Materials**—Dithiothreitol, FMN, cAMP, decyl aldehyde, bovine serum albumin (fraction V), pyruvate, glucose-1,6-P₂, vasopressin, (−)-phenylephrine, (−)-epinephrine, d-fructose, d-tagatose, L-sorbose, D-glyceraldehyde, dihydroxyacetone, L-lactate, D-galactose, xylose, D-arabinose, D-ribose, and carbonyl cyanide m-chlorophenylhydrazine were from Sigma. Glucose-6-P, fructose-6-P, fructose-1,6-P₂, NAD, NADH, phosphoglucone isomerase, glucose-6-phosphate dehydrogenase (from *Leuconostoc mesenteroides*) and lactate dehydrogenase were from Boehringer Mannheim. Luciferase (from *Photobacterium fischeri*) and collagenase were from Worthington. Glucagon, insulin, and A23187 ionophore were gifts from Lilly. D-Glucose was from Fisher; glycerol was from J. T. Baker Chemical Co.; D-Mannose was from Nutritional Biochemicals; and L-Alanine was from Mann.

Fructose-2,6-P₂ was a gift of Dr. E. Van Schaftingen, Brussels; it was prepared from fructose-1,6-P₂ by a modification of the procedure of Pontis and Fischer (24, 25).

**RESULTS**

**Fructose-2,6-P₂ in Isolated Rat Hepatocytes**—The new “enzymatic” method was applied to isolated hepatocytes from fed rats and the results shown in Table I are compared with those obtained with the “stimulation assay” method based on the ability of fructose-2,6-P₂ to stimulate phosphofructokinase activity (6). It is seen that the effects of glucose, phenylephrine, vasopressin, and the ionophore A23187 to increase fruc-
Regulation of Fructose-2,6-bisphosphate Levels

Fructose-2,6-P$_2$ content of hepatocytes incubated with various substrates or hormones

Hepatocytes from fed rats were incubated for 15 min in the presence of the various substrates or hormones. Values shown are means ± S.E. (number of cell preparations). Values in λ were obtained with the method described in this article whereas values in B were obtained with the stimulation assay and are taken from (6).

Table I

<table>
<thead>
<tr>
<th>Substrate or Hormone</th>
<th>A (nmol/g of cells)</th>
<th>B (nmol/g of cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>1.9 ± 0.7 (6)</td>
<td>1.4 ± 0.7 (6)</td>
</tr>
<tr>
<td>Glucagon, 10$^{-8}$ M</td>
<td>5.7 ± 0.2 (3)</td>
<td>3.8 ± 0.2 (3)</td>
</tr>
<tr>
<td>Adenosine, 10$^{-4}$ M</td>
<td>5.1 ± 0.1 (1)</td>
<td>4.2 ± 0.1 (1)</td>
</tr>
<tr>
<td>Phosphoenolpyruvate, 10$^{-3}$ M</td>
<td>7.4</td>
<td>5.0 ± 0.2 (6)</td>
</tr>
<tr>
<td>A23187, 10$^{-5}$ M</td>
<td>6.7 ± 1.2 (3)</td>
<td>7.4 ± 0.8 (4)</td>
</tr>
<tr>
<td>A-D-Fructose, 20 mM</td>
<td>0.3 ± 0.1 (4)*</td>
<td>0.3 ± 0.1 (4)*</td>
</tr>
<tr>
<td>D-Tagatose, 20 mM</td>
<td>0.4 ± 0.1 (5)*</td>
<td>0.4 ± 0.1 (5)*</td>
</tr>
<tr>
<td>L-Sorbitose, 20 mM</td>
<td>0.8 ± 0.3 (5)*</td>
<td>0.8 ± 0.3 (5)*</td>
</tr>
<tr>
<td>D-L-Glyceraldehyde, 10 mM</td>
<td>1.0 ± 0.2 (4)*</td>
<td>1.0 ± 0.2 (4)*</td>
</tr>
<tr>
<td>Glyceraldehyde, 20 mM</td>
<td>1.0 ± 0.4 (4)*</td>
<td>1.0 ± 0.4 (4)*</td>
</tr>
<tr>
<td>Dihydroxyacetone, 20 mM</td>
<td>4.1 ± 0.9 (5)</td>
<td>4.1 ± 0.9 (5)</td>
</tr>
<tr>
<td>Pyruvate, 20 mM</td>
<td>1.6 ± 0.5 (3)*</td>
<td>1.6 ± 0.5 (3)*</td>
</tr>
<tr>
<td>L-Lactate, 20 mM</td>
<td>0.5 ± 0.2 (4)*</td>
<td>0.5 ± 0.2 (4)*</td>
</tr>
<tr>
<td>L-Alanine, 20 mM</td>
<td>1.8 ± 0.6 (3)*</td>
<td>1.8 ± 0.6 (3)*</td>
</tr>
<tr>
<td>N$_2$</td>
<td>2.3 ± 0.7 (3)*</td>
<td>2.3 ± 0.7 (3)*</td>
</tr>
<tr>
<td>Carboxylin cyanide-</td>
<td>3.4 ± 0.3 (3)*</td>
<td>3.4 ± 0.3 (3)*</td>
</tr>
<tr>
<td>chlorofluorohydrinze</td>
<td></td>
<td></td>
</tr>
<tr>
<td>d-Glucose, 50 mM</td>
<td>2.3 ± 0.5 (3)*</td>
<td>2.3 ± 0.5 (3)*</td>
</tr>
<tr>
<td>D-Galactose, 20 mM</td>
<td>4.5 ± 1.7 (5)</td>
<td>4.5 ± 1.7 (5)</td>
</tr>
<tr>
<td>D-Mannose, 20 mM</td>
<td>2.7 ± 0.3 (5)*</td>
<td>2.7 ± 0.3 (5)*</td>
</tr>
<tr>
<td>Xylitol, 20 mM</td>
<td>3.6 ± 0.7 (5)*</td>
<td>3.6 ± 0.7 (5)*</td>
</tr>
<tr>
<td>d-Arabinose, 20 mM</td>
<td>4.0 ± 1.1 (4)</td>
<td>4.0 ± 1.1 (4)</td>
</tr>
<tr>
<td>D-Ribose, 20 mM</td>
<td>2.7 ± 0.7 (3)</td>
<td>2.7 ± 0.7 (3)</td>
</tr>
</tbody>
</table>

Note: p < 0.05 compared with control by one-tailed paired t test.

Fructose, D-Fructose, and D-Sorbitose were added to the incubation medium at variable concentrations of 5-100 mM. The results are shown in Table I.

Incubation of hepatocytes with relatively high concentrations (20 mM) of D-galactose, D-Mannose, and D-Ribose produced an increase in fructose-2,6-P$_2$ which was abolished by 2.5 mM D-Tagatose. The addition of D-Sorbitose caused a decrease in fructose-2,6-P$_2$. The addition of glycerol caused an increase in fructose-2,6-P$_2$. The addition of D-Glucose caused an increase in fructose-2,6-P$_2$ and a decrease in fructose-2,6-P$_2$.

Incubation of hepatocytes from fed rats in an atmosphere of N$_2$ or in the presence of carbonyl cyanide m-chlorophenylhydrazone, a mitochondrial uncoupler (27), produced an increase in fructose-2,6-P$_2$.

Fructose-2,6-P$_2$ content of various rat tissues

Fed rats were anesthetized (60 mg/kg of Na pentobarbital intraperitoneally) and tissue samples were freeze-clamped. Values shown are means ± S.E. (number of samples). Values in parentheses are values obtained with the stimulation assay and are taken from (6).

Table II

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Fructose-2,6-P$_2$ (nmol/g of tissue)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Skeletal muscle</td>
<td>0.43 ± 0.08 (5)</td>
</tr>
<tr>
<td>Heart</td>
<td>3.52 ± 0.02 (4)</td>
</tr>
<tr>
<td>Liver</td>
<td>2.04 ± 0.04 (4)</td>
</tr>
<tr>
<td>Liver, after glucose load*</td>
<td>7.88 (2)</td>
</tr>
<tr>
<td>Kidney</td>
<td>0.22 (2)</td>
</tr>
<tr>
<td>Brain</td>
<td>2.29 ± 0.6 (3)</td>
</tr>
<tr>
<td>Epididymal fat</td>
<td>0.11 (2)</td>
</tr>
<tr>
<td>Lung</td>
<td>0.54 ± 0.15 (4)</td>
</tr>
</tbody>
</table>

* 3 g/kg injected intravenously 10 min before sampling.
The present report confirms earlier observations (4-8) showing that, in hepatocytes from fed rats, glucose, vasopressin, phenylephrine, and the ionophore A23187 cause an increase in fructose-2,6-P₂ content, whereas glucagon has the opposite effect (4-8).

A new finding is the drastic decrease in fructose-2,6-P₂ observed in hepatocytes incubated with 20 mM keto-hexoses, glycercor, or glyceraldehyde. One may speculate that the decreased concentration results from the fact that high concentrations of all these compounds have in common the property of decreasing ATP (26) which is a substrate for the enzyme synthesizing fructose-2,6-P₂ from fructose-6-P in the liver (6, 34). In addition, it is possible that ester phosphates, such as ketose-1-phosphate and sn-glycerol-3-P which accumulate during the metabolism of these sugars (28), inhibit the synthesis of fructose-2,6-P₂. A stimulation of degradation of the metabolite is not excluded. High concentrations of fructose have been reported to increase cAMP content in perfused livers (35) which may, in turn, lead to an inactivation of the enzyme synthesizing fructose-2,6-P₂ (6, 36).

The decrease in fructose-2,6-P₂, observed after 10-20 mM fructose would be expected to increase metabolic flux in the gluconeogenetic direction at the level of the fructose-6-P/fructose-1,6-P₂ cycle. However, it is well known that the metabolism of fructose results in a large production of lactate, sometimes referred to as “fructolysis” (26). Indeed, fructose enters the glycolytic pathway at the level of triose phosphates and the production of lactate from fructose does not involve phophofructokinase. The other metabolic changes produced by high concentrations of fructose, e.g. the change in adenine nucleotides and the rise in fructose-1,6-P₂ (26, 37), may override the effects of the decrease in fructose-2,6-P₂ on phosphofructokinase and also stimulate glycogenic flux at the level of pyruvate kinase (38, 39).

Another remarkable feature of Table I is the effect of lactate, pyruvate, and alanine to decrease fructose-2,6-P₂ content. Here again one may speculate that the synthesis of fructose-2,6-P₂ from fructose-6-P is inhibited by P-enolpyruvate which accumulates in livers perfused with these substrates (38, 40, 41). As a result of the decrease in fructose-2,6-P₂, gluconeogenesis is favored and the glycolytic flux is inhibited at the level of phosphofructokinase. However, it should be noted that these effects may be obtained with relatively high doses of precursors and lower doses may not necessarily have the same effect.

The acceleration of glycolysis is a well known feature of liver during anoxia (28). Under this condition the concentration of fructose-2,6-P₂ is decreased, indicating that other mechanisms, possibly related to adenine nucleotide changes, are involved in the effect.

**Discussion**

**Method of Measurement of Fructose-2,6-P₂**—The enzymatic method for the measurement of fructose-2,6-P₂ described here has the advantage of being more specific than the previous one (4, 5). Indeed, the existing assay based on the ability of fructose-2,6-P₂ to stimulate phosphofructokinase activity can be affected in various ways. The tissue extracts contain several metabolites such as ATP, AMP, P₃, glucose-1,5-P₃, fructose-1,6-P₂, triose-P, etc., which can interfere with the assay of phosphofructokinase activity. If the concentration of these metabolites were changed by the experimental conditions under study, the assay for fructose-2,6-P₂ could become unreliable. Such large metabolite changes occur in extracts of hepatocytes which have been incubated under anoxic conditions or with keto sugars and in exercising or anoxic muscle preparations.

The specificity of the method developed here is based on the remarkable acid lability of fructose-2,6-P₂. More than 95% of fructose-2,6-P₂ is hydrolyzed in 10 min at pH 2.0 at room temperature, whereas less than 1% of fructose-1,6-P₂ and glucose-1,6-P₂ is hydrolyzed under these conditions (2, 3). The enzymatic method requires that the endogenous fructose-6-P and glucose-6-P should be removed. This is achieved by the glucose-6-P dehydrogenase and lactate dehydrogenase coupled system and by purification on Dowex AG-1X8. After these steps, hexose-6-P is almost undetectable.

**Fructose-2,6-P₂ in Isolated Hepatocytes from Fed Rats**—The present report confirms earlier observations (4-8) showing that, in hepatocytes from fed rats, glucose, vasopressin, phenylephrine, and the ionophore A23187 cause an increase in fructose-2,6-P₂ content, whereas glucagon has the opposite effect (4-8).

**Table III**

<table>
<thead>
<tr>
<th>Hexose-6-P and fructose-2,5-P₂ content of perfused rat hindlimbs</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Values shown are means ± S.E. (number of preparations). Hexose-6-P values are the sum of the glucose-6-P and fructose-6-P content.</td>
<td></td>
</tr>
<tr>
<td>Conditions of perfusion</td>
<td>Hexose-6-P</td>
</tr>
<tr>
<td>---</td>
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</tr>
<tr>
<td>Control</td>
<td>0.40 ± 0.05 (5)</td>
</tr>
<tr>
<td>Electrical stimulation</td>
<td>2.06 ± 0.96 (3)</td>
</tr>
<tr>
<td>Epinephrine 10⁻⁷ M</td>
<td>2.35 ± 0.10 (5)</td>
</tr>
<tr>
<td>Epinephrine + electrical stimulation</td>
<td>6.50 ± 0.15 (3)</td>
</tr>
<tr>
<td>Insulin 6 nM</td>
<td>0.45 ± 0.04 (5)</td>
</tr>
</tbody>
</table>

**Table IV**

<table>
<thead>
<tr>
<th>Fructose-2,6-P₂ content, protein kinase, and phosphofructokinase activities in perfused rat hearts</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Values shown are means ± S.E. (number of preparations).</td>
<td></td>
</tr>
<tr>
<td>Conditions of perfusion</td>
<td>Fructose-2,6-P₂</td>
</tr>
<tr>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>Control</td>
<td>0.67 ± 0.08 (4)</td>
</tr>
<tr>
<td>Epinephrine, 5 µM (2 min)</td>
<td>0.57 ± 0.20 (4)</td>
</tr>
<tr>
<td>Epinephrine, 5 µM (5 min)</td>
<td>0.74 ± 0.07 (8)</td>
</tr>
</tbody>
</table>

Therefore studied in hearts perfused with 5 µM epinephrine for various periods of time. Increases in the activity ratios of phosphofructokinase (−cAMP/+cAMP) and of protein kinase (−cAMP/+cAMP) were taken as indices of the efficacy of the epinephrine treatment. Table IV shows that epinephrine was without effect on fructose-2,6-P₂ at 2 or 5 min, although it caused an activation of both protein kinase and phosphofructokinase.
in fructose-2,6-P in view of the extreme acid lability of this compound.

In perfused hearts, epinephrine caused an activation of the cyclic AMP-dependent protein kinase and phosphorylase. Although lactate production was not measured in the present study, many studies have shown a large increase with epinephrine (e.g. Ref. 46). However, the hormone did not increase the fructose-2,6-P$_2$ content, indicating that this metabolite is not involved in the stimulation of glycolysis by epinephrine in this tissue.

Role of Fructose-2,6-P$_2$ in the Regulation of Glycolysis—Evidence presented here and elsewhere (4–6) shows that, in isolated hepatocytes, an increased concentration of fructose-2,6-P$_2$ is often accompanied by increased glycolysis. However, it is also evident that increased glycolysis can occur in liver and other tissues with no concomitant increase in fructose-2,6-P$_2$. This was observed in hepatocytes during anoxia, in muscles during electrical stimulation, and in hearts treated with epinephrine. Obviously, mechanisms other than changes in fructose-2,6-P$_2$ concentration must then be involved in the regulation of glycolysis under these conditions. A major consideration is whether or not changes in fructose-2,6-P$_2$ have physiological significance for metabolic processes other than hepatic glycolysis and gluconeogenesis. The intriguing possibility that this compound exerts effects on other enzymes besides phosphofructokinase and fructose 1,6-bisphosphatase needs to be actively explored.

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21. Deleted in proof
Regulation of fructose-2,6-bisphosphate content in rat hepatocytes, perfused hearts, and perfused hindlimbs.
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