The inhibition of hepatocyte 6-phosphofructo-1-kinase by glucagon was suppressed by insulin when the enzyme was measured in crude extracts. However, no effect of either hormone was observed after the removal of allosteric effectors from the enzyme, suggesting that the alterations in activity may be due to changes in the level of fructose 2,6-bisphosphate, a potent allosteric activator of the enzyme. Insulin opposed the action of both glucagon and exogenous cyclic AMP to lower fructose 2,6-bisphosphate levels. The concentration of glucagon and of cyclic AMP that gave a half-maximal decrease in fructose 2,6-bisphosphate levels was increased in the presence of 10 nM insulin from 0.03 to 0.09 nM and from 12 to 36 µM, respectively. Insulin also counteracted the effect of maximal concentrations of epinephrine on fructose 2,6-bisphosphate levels. In the presence of 0.02 nM glucagon or 10 µM epinephrine, 10 nM insulin enhanced 6-phosphofructo-2-kinase and decreased fructose 2,6-bisphosphatase activity in (NH₄)₂SO₄-treated hepatocyte extracts. The bifunctional enzyme 6-phosphofructo-2-kinase/fructose 2,6-bisphosphatase was shown to be a substrate for the cAMP-dependent protein kinase but not for phosphorylase kinase. It was concluded that insulin opposed the action of glucagon and epinephrine by affecting the phosphorylation state of 6-phosphofructo-2-kinase/fructose 2,6-bisphosphatase.

Fructose 2,6-bisphosphate levels were decreased in liver cells from diabetic rats. Addition of 30 mM glucose elevated fructose 2,6-bisphosphate levels in cells from fed and 24-h-starved rats but not in cells from diabetic rats. This was probably due to decreases in both 6-phosphofructo-2-kinase and glucokinase activity in the diabetic state. These results show that insulin has both short and long term effects on fructose 2,6-bisphosphate metabolism in liver.

Evidence from a number of laboratories has implicated fructose 2,6-bisphosphate as an important factor in the regulation of hepatic glycogenolysis and gluconeogenesis by glucagon (1–16). The action of glucagon in lowering the level of this compound is rapid and has been shown to be due, at least in part, to a cAMP-dependent protein kinase-catalyzed phosphorylation of the enzyme responsible for the synthesis and degradation of the compound (1, 11, 17, 18). Although insulin has been shown to oppose the action of glucagon on gluconeogenesis (19, 20), at least in part by modulating the phosphorylation of pyruvate kinase (21), only recently has evidence begun to accumulate that insulin affects fructose 2,6-bisphosphate metabolism. The level of fructose 2,6-bisphosphate has been shown to be decreased in both starvation and diabetes, and refeeding and insulin administration, respectively, restore the level to normal (22). However, there is as yet no evidence that insulin can act acutely (i.e., within minutes) to modulate the level of fructose 2,6-bisphosphate. The object of this study was to determine if insulin can oppose the action of glucagon to lower fructose 2,6-bisphosphate levels in isolated hepatocytes from fed rats and if so to investigate the site of action of the hormone. We also investigated the acute regulation of fructose 2,6-bisphosphate levels by hormones and substrate during starvation and diabetes.

**MATERIALS AND METHODS**

**Preparation and Incubation of Isolated Hepatocytes—**Isolated hepatocytes were prepared from fed rats (male, Sprague-Dawley, 175–225 g) as previously described (10). The cells were suspended in a final concentration of 50 mg of liver (wet weight)/ml in Krebs-Henseleit bicarbonate buffer that contained 0.5% bacitracin (to minimize hormone degradation) and incubated without any additions for 20 min at 37 °C. Cells (approximately 0.5–0.5 g of liver/flask) were incubated in 50-ml flasks with varying concentrations of hormones for 10 min and then rapidly centrifuged. The cells were homogenized for 90 s (30 s, three times) with an Ultraturax homogenizer in 10 ml of cold homogenizing buffer that contained 50 mM N-tris[hydroxymethyl]methyl-2-amino)ethanesulfonic acid, pH 7.5, 50 mM KCI, 5 mM MgCl₂, 2 mM EDTA, 1 mM dithiothreitol, 0.2 mM phenylmethylsulfonyl fluoride, 5 µg/ml leupeptin, and the homogenate was centrifuged at 30,000 × g for 30 min.

Enough solid (NH₄)₂SO₄ was added to the supernatant fraction to make it 30% saturated and the precipitate was dissolved in 1 ml of Buffer A which contained 20 mM N-tris[hydroxymethyl]methyl-2-amino)ethanesulfonic acid, pH 7.5, 50 mM KCl, 5 mM MgCl₂, 2 mM EDTA, 1 mM dithiothreitol, 0.2 mM phenylmethylsulfonyl fluoride. 6-Phosphofructo-1-kinase activity was found in this fraction. The supernatant fraction was then made 70% saturated with (NH₄)₂SO₄ and the precipitate was resuspended in 1 ml of Buffer A and the (NH₄)₂SO₄ was removed by dialysis against the same buffer. The (NH₄)₂SO₄-treated hepatocyte extracts were then assayed for 6-phosphofructo-2-kinase, 6-phosphofructo-1-kinase, pyruvate kinase, and fructose 2,6-Pase activity. In some instances 6-phosphofructo-1-kinase activity was measured in the hepatocyte extracts prior to (NH₄)₂SO₄ fractionation.

**Determination of Fructose 2,6 Bisphosphate in Hepatocytes—**Hepatocytes were rapidly sedimented and then homogenized for 30 s in 50 mM triethylamine HCl buffer, pH 8.5, containing 0.1 mM EDTA at 90 °C. The homogenate was heated at 90 °C for 5 min, cooled, and centrifuged, and the supernatant was treated with charcoal to remove nucleotides. The amount of fructose 2,6-bisphosphate was determined with the 6-phosphofructo-1-kinase activation assay.

**6-Phosphofructo-1-kinase Activation Assay for Fructose 2,6-Bisphosphate—**Fructose 2,6-bisphosphate was assayed by comparing the amount of activation of 6-phosphofructo-1-kinase obtained with base-
treated samples with the activation obtained with known concentrations of fructose 2,6-bisphosphate as previously described (1, 11, 22).

Fructose 2,6-bisphosphate was prepared by the method of Pilks et al. (2). The concentration of the synthetic compound was determined by incubating aliquots at pH 3.0 for 30 min and assaying the amount of fructose 6-phosphate formed (2).

Assay of 6-Phosphofructo-2-kinase and Fructose 2,6-Bisphosphatase—6-Phosphofructo-2-kinase activity was measured by following the production of fructose 2,6-bisphosphate with the 6-phosphofructo-1-kinase activation assay as previously described (1, 11, 23). One unit of enzyme is defined as the amount that catalyzes the synthesis of 1 pmol of fructose 2,6-bisphosphate/min.

Fructose 2,6-bisphosphatase activity was measured by following the disappearance of fructose 2,6-bisphosphate with the 6-phosphofructo-1-kinase activation assay as previously described (1, 23). One unit of enzyme is the amount that catalyzes the disappearance of 1 pmol of fructose 2,6-bisphosphate/min.

Preparation of Phosphorylase Kinase—Rat liver phosphorylase kinase was purified according to the method of Chrisman et al. (25), in which the inhibitor of the cAMP-dependent protein kinase was purified according to the method of Ashby and Walsh (26), and the catalytic subunit of the beef heart cAMP-dependent protein kinase was a gift from Dr. C. Brostrom (Vanderbilt University, Nashville, TN). Rabbit skeletal muscle phosphorylase kinase was prepared by the method of Brostrum et al. (27) and further purified by DEAE-cellulose chromatography (28) and rabbit skeletal muscle glycogen phosphorylase b was a gift from Dr. E. G. Krebs (University of Washington, Seattle, WA). Calmodulin was prepared from porcine brain by the method of Vanaman and Watterson (29).

Protein Kinase catalyzed Phosphorylation of Hepatic 6-Phosphofructo-2-kinase/Fructose 2,6-Bisphosphatase—For all incubations (10 min, 30°C) the 50-μl reaction mixture contained 50 μM EDTA, 50 mM KCl. The 6-phosphofructo-2-kinase/fructose 2,6-bisphosphatase used was a homogeneous preparation from rat liver (24). Incubations with catalytic subunit of the cAMP-dependent protein kinase (280 microunits) contained 6.25 μg of 6-phosphofructo-2-kinase/fructose 2,6-bisphosphatase, 20 mM 2-(N-morpholino)ethanesulfonic acid (pH 6.8), 1 mM magnesium acetate, 0.5 mM [γ-32P]ATP (167 cpm/pmol), and, where indicated, an excess of the heat-stable inhibitor of the catalytic subunit. When using skeletal muscle phosphorylase kinase (1.4 pg), the incubation mixture contained 20 μM Tris-HCl (pH 8.2), 4 mM magnesium acetate, 1 mM [γ-32P]ATP (312 cpm/pmol), the heat-stable inhibitor of the catalytic subunit 0.2 mM CaCl2, 0.95 μg of calmodulin, and, where indicated, 6.25 μg of 6-phosphofructo-2-kinase/fructose 2,6-bisphosphatase or 6.25 μg of glycogen phosphorylase b. When rat liver phosphorylase kinase (0.13 μg) was used, the incubation mixture contained 20 mM 2-(N-morpholino)ethanesulfonic acid (pH 6.8), 1 mM magnesium acetate, 0.5 mM [γ-32P]ATP (167 cpm/pmol), the heat-stable inhibitor of the catalytic subunit, and, where indicated, 6.25 μg of 6-phosphofructo-2-kinase/fructose 2,6-bisphosphatase or 6.25 μg of glycogen phosphorylase b.

The kinase reaction was initiated with Mg[y-32P]ATP and terminated with 200 μl of cold 10% trichloroacetic acid, 10 mM ATP, kept at 4°C for 30 min, and centrifuged briefly, and the supernatant was removed by aspiration.

Electrophoresis in 6% polyacrylamide vertical slab gels in the presence of 0.1% sodium dodecyl sulfate was performed at pH 8.5 (30) using a 4% spacer gel (pH 6.8). The trichloroacetic acid-precipitated protein was dissolved in 50 μl of sample buffer containing Tris-glycine (0.025 M, 0.19 M, pH 8.5), 0.017% bromphenol blue, 2% sodium dodecyl sulfate, 2% mercaptoethanol, 8% percent sucrose and heated 5 min at 100°C. The gels were fixed and stained in 40% methanol, 7% acetic acid, 0.2% Coomassie blue R-250 and destained by diffusion in 40% methanol, 7% acetic acid. Autoradiograms were made by exposing the dried Coomassie blue-stained gels to DuPont Cronex x-ray film.

Other Methods—Protein was determined by the method of Lowry et al. (31) with bovine serum albumin as the standard. Pyruvate kinase activity was assayed as previously described (21) as was the level of fructose 1,6-bisphosphate (21).

RESULTS

Effect of Insulin on the Activity of Hepatocyte 6-Phosphofructo-1-kinase—Addition of glucagon to isolated hepatocytes results in inactivation of 6-phosphofructo-1-kinase when activity is measured in crude extracts (6-8) but not when fructose 2,6-bisphosphate is removed by (NH4)2SO4 precipitation of the enzyme (1-9). It was of interest to determine if insulin had any effect on 6-phosphofructo-1-kinase activity. Fig. 1 shows the dose-response curves for the inhibition of 6-phosphofructo-1-kinase activity by glucagon in the absence and presence of 10 nM insulin. Insulin had a slight stimulatory effect on the activity of the enzyme measured in the absence of glucagon and abolished changes in activity induced by low concentrations of glucagon. As the glucagon concentration increased, insulin became less effective and, at concentrations of glucagon of 1 nM or higher, insulin had no effect. The concentration of glucagon that gave a half-maximal inactivation of 6-phosphofructo-1-kinase was increased from about 0.15 to 0.5 nM in the presence of insulin. Recent evidence strongly suggests that the inactivation of the 6-phosphofructo-1-kinase by glucagon is due in large part to the hormone-induced decrease in the level of fructose 2,6-bisphosphate, a potent activator of the enzyme (1-9). In order to determine if this was also true for the effect of insulin on activity, the enzyme was partially purified from the crude extracts. Ammonium sulfate precipitation (0-30%) abolished the effect of both glucagon and insulin (Fig. 1). Since treatment with...
(NH₄)₂SO₄ removes fructose 2,6-P₂ from the enzyme (16), the present results suggest that insulin may oppose the action of glucagon-induced inhibition of 6-phosphofructo-1-kinase activity by countering the effect of glucagon to lower fructose 2,6-bisphosphate levels.

Effect of Insulin on the Hepatic Level of Fructose 2,6-Bisphosphate—Fig. 2A shows the dose-response curves for the glucagon-induced decrease in hepatocyte fructose 2,6-bisphosphate levels in the absence or presence of 10 nM insulin. Insulin suppressed the decrease in the level of the compound brought about by low concentrations of hormone. As the glucagon concentration increased further, insulin became less effective until it had no effect at glucagon concentrations of 0.7 nM or greater (data not shown). The presence of insulin increased the concentration of glucagon needed to obtain a half-maximal decrease in fructose 2,6-bisphosphate levels from about 0.03 to 0.09 nM, but insulin had no effect when added alone.

Similar results were obtained when exogenous cyclic AMP was used to lower the level of fructose 2,6-bisphosphate (Fig. 2B). Insulin completely opposed the effect of concentrations of the nucleotide of 10 μM or less. Further increase in the cyclic AMP concentration resulted in a diminished insulin inhibition until at about 0.5 μM cyclic AMP no inhibition was observed. Thus, insulin increased the concentration of cyclic AMP necessary for a half-maximal decrease in fructose 2,6-bisphosphate levels from 12 to 36 μM.

Fig. 3 shows that the effect of a submaximal concentration of glucagon (0.02 nM) on the level of fructose 2,6-bisphosphate can be opposed by increasing concentrations of insulin. Half-maximal effects of the hormone were seen with about 0.5 nM insulin. The effects of both insulin and glucagon did not require protein synthesis since the effects were seen in hepatocytes incubated with cycloheximide where protein synthesis was inhibited greater than 95% (data not shown).

Effect of Catecholamines and Insulin on the Level of Fructose 2,6-Bisphosphate in Hepatocytes from Fed Rats—High concentrations of epinephrine have been reported to depress fructose 2,6-bisphosphate levels in hepatocytes (32). Fig. 4 shows the time course of the effect of maximally effective concentrations of epinephrine (10 μM) as well as glucagon on hepatocyte fructose 2,6-bisphosphate levels. A maximal effect of epinephrine is seen at a concentration of 2 μM. No effect of epinephrine was observed for 5 min, while 10 nM glucagon caused a greater than 90% decrease in the level of the compound within 1 min. Only after 15 min of exposure...
to epinephrine did the level of fructose 2,6-bisphosphate approach that seen with glucagon after 1 min. These findings confirm the observations of Richards et al. (32). Addition of 10 nM insulin opposed the action of epinephrine. The demonstration that insulin is able to oppose the action of maximal concentrations of epinephrine on fructose 2,6-bisphosphate levels is similar to the previous finding that insulin opposed the effect of maximal concentrations of epinephrine to stimulate lactate gluconeogenesis (20).

The nature of the catecholamine effect on fructose 2,6-bisphosphate levels in isolated hepatocytes is uncertain (9, 10, 17, 18). In order to determine whether catecholamines lowered fructose 2,6-bisphosphate levels by an α-adrenergic mechanism, we examined the ability of norepinephrine and of epinephrine in the presence of propranolol to modulate fructose 2,6-bisphosphate levels in hepatocytes prepared from fed rats. These agents have been shown to enhance glycogenolysis and gluconeogenesis by a Ca\(^{2+}\)-dependent mechanism which is associated with the phosphorylation of a number of specific proteins in the hepatocyte (33, 34). These agents, at concentrations up to 10 μM of agonist plus propranolol (1 μM), had no effect on the level of fructose 2,6-bisphosphate or on intracellular cAMP levels (data not shown).

**Effect of Insulin on Gluconeogenesis from 2 mM [U-\(^{14}C\)]Lactate and on Pyruvate Kinase Activity at Varied Glucagon and cAMP Concentrations**—The results in Figs. 2 and 3 show that the level of fructose 2,6-bisphosphate in hepatocytes from fed rats is sensitive to the addition of low concentrations of glucagon or cAMP. It was of interest to determine if these low concentrations of hormone and cyclic AMP would affect gluconeogenesis or pyruvate kinase activity in hepatocytes. Table I summarizes the effects of glucagon and cyclic AMP, in the absence and presence of 10 nM insulin, on gluconeogenesis from 2 mM [U-\(^{14}C\)]lactate, pyruvate kinase activity, and the level of fructose 2,6-bisphosphate (data from Fig. 2). The data on gluconeogenesis and pyruvate kinase are taken from previously published work (20, 21). The concentration of glucagon necessary for a half-maximal stimulation of glucose synthesis and a half-maximal inactivation of pyruvate kinase was about 0.3–0.4 nM in the absence of insulin and about 0.6–0.9 nM in the presence of insulin. Glucagon stimulated glucose synthesis and inhibited pyruvate kinase activity only at concentrations of hormone above 0.1 nM while this concentration caused a maximal decrease in the level of fructose 2,6-bisphosphate. Glucagon-induced stimulation of gluconeogenesis and inactivation of pyruvate kinase correlated almost perfectly with one another with regard to dependence on glucagon concentration. However, by comparison of Table I with Fig. 2, it is clear that the level of fructose 2,6-bisphosphate is about an order of magnitude more sensitive to glucagon action than either gluconeogenesis or pyruvate kinase activity.

**The Effect of Insulin on 6-Phosphofructo-2-kinase and Fructose 2,6-Bisphosphatase Activity**—The ability of insulin to oppose the action of glucagon and cyclic AMP on fructose 2,6-bisphosphate levels suggests that insulin acts by lowering the level of cyclic AMP (19, 20). Since both 6-phosphofructo-2-kinase and fructose 2,6-bisphosphatase have been shown to be regulated by cyclic AMP-dependent phosphorylation (23, 24), it might be expected that insulin would affect one or both of these activities. Table II shows the effect of 10 nM insulin on the activity of these enzymes measured in (NH\(_4\))\(_2\)SO\(_4\)-treated extracts of cells incubated with and without 0.02 nM glucagon. Addition of 0.02 nM glucagon caused an inactivation of 6-phosphofructo-2-kinase and an activation of fructose 2,6-bisphosphatase as has been reported previously (1, 18, 23). The ratio of the phosphotransferase to the phosphorylase activity decreased from 4.6 in the control to 0.2 with glucagon. Insulin (10 nM) opposed the action of glucagon on both activities, increasing the ratio of the two activities to 1.4, but had no effect when added alone. Table II also shows that the addition of 10 μM epinephrine resulted in a decrease in 6-phosphofructo-2-kinase activity and an increase in fructose 2,6-bisphosphatase activity, with a decrease in the ratio of the two activities to 0.7. Insulin opposed the effect of epinephrine on both activities, increasing the ratio of phosphotransferase to phosphorylase to 2.0. In this experiment, the enzyme activities were measured with near physiological substrate concentrations and the ratio of phosphotransferase to phosphorylase activity is probably a reasonable index of net fructose 2,6-bisphosphate production.

**The Role of Hormones in Regulation of the Phosphorylation State of 6-Phosphofructo-2-kinase/ Fructose 2,6-Bisphosphatase**—Since insulin opposes the effect of glucagon at concentrations which have little or no effect on the level of cyclic AMP (19, 20) or on inactivation of pyruvate kinase (Table I), this suggests either that insulin and glucagon act on 6-phosphofructo-2-kinase/fructose 2,6-bisphosphatase by a cyclic AMP-dependent mechanism where only very small changes in cyclic AMP are necessary to modulate the phosphorylation state of these enzymes or that the hormones act by a cyclic AMP-independent mechanism. We have purified 6-phosphofructo-2-kinase/fructose 2,6-bisphosphatase to homogeneity and have shown that the purified protein has both 6-phosphofructo-2-kinase and fructose 2,6-bisphosphatase activities, i.e.

**Table I**

<table>
<thead>
<tr>
<th>Glucagon</th>
<th>Pyruvate kinase activity</th>
<th>Fructose 2,6-P&lt;sub&gt;1&lt;/sub&gt;</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucagon, nM</td>
<td>0.3</td>
<td>0.3</td>
<td>0.03</td>
</tr>
<tr>
<td>Glucagon, nM + insulin, 10 nM</td>
<td>0.8</td>
<td>0.6</td>
<td>0.09</td>
</tr>
<tr>
<td>Cyclic AMP, μM</td>
<td>35</td>
<td>62</td>
<td>12</td>
</tr>
<tr>
<td>Cyclic AMP, nM + insulin, 10 nM</td>
<td>110</td>
<td>120</td>
<td>36</td>
</tr>
</tbody>
</table>

**Table II**

<table>
<thead>
<tr>
<th>Condition</th>
<th>6-Phosphofructo-2-kinase</th>
<th>Fructose 2,6-bisphosphatase</th>
<th>Concentration ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>No additions</td>
<td>1.10</td>
<td>0.24</td>
<td>4.6</td>
</tr>
<tr>
<td>Glucagon, 0.02 nM</td>
<td>0.20</td>
<td>0.94</td>
<td>0.2</td>
</tr>
<tr>
<td>Insulin, 10 nM</td>
<td>1.20</td>
<td>0.18</td>
<td>6.7</td>
</tr>
<tr>
<td>Glucagon, 0.02 nM + insulin, 10 nM</td>
<td>0.63</td>
<td>0.46</td>
<td>1.4</td>
</tr>
<tr>
<td>Epinephrine, 10 μM</td>
<td>0.56</td>
<td>0.76</td>
<td>0.7</td>
</tr>
<tr>
<td>Epinephrine, 10 μM + insulin, 10 nM</td>
<td>0.77</td>
<td>0.39</td>
<td>2.0</td>
</tr>
</tbody>
</table>
that it is bifunctional, and that the protein is a very good substrate for the cyclic AMP-dependent protein kinase (23, 24). Two-dimensional sodium dodecyl sulfate-gel electrophoresis of the phosphorylated and nonphosphorylated form of the enzyme is shown in Fig. 5. The nonphosphorylated peptide migrated as a Coomassie blue-staining spot with a subunit Mr of 55,000 and a pl equal to about 6.6.\(^1\) Phosphorylation of the protein catalyzed by the catalytic subunit of the cAMP-dependent protein kinase resulted in a charge shift to pH 6.4. At zero time, the enzyme is primarily in the nonphosphorylated form while at 5 min, the nonphosphorylated form is beginning to disappear, and at 15 min, all of the enzyme is phosphorylated.

Comparison of the migration position and charge shift upon phosphorylation of the purified enzyme with that of \(^32\)P-phosphopeptides observed in extracts of whole cells incubated with \(^32\)P in the presence and absence of glucagon revealed that the 6-phosphofructo-2-kinase/fructose 2,6-bisphosphatase phosphorylated by the catalytic subunit of cAMP-dependent protein kinase, rabbit skeletal muscle phosphorylase kinase, and rat liver phosphorylase kinase. Incubation conditions, electrophoresis in 6% polyacrylamide, 0.1% sodium dodecyl sulfate, staining, and autoradiography were as described under "Materials and Methods." Lane A1, Coomassie blue stain of 6.25 \(\mu\)g of 6-phosphofructo-2-kinase/fructose 2,6-bisphosphatase; lane A2, Coomassie blue stain of 20 \(\mu\)g of rabbit skeletal muscle phosphorylase kinase. Lane B3, autoradiogram of 6.25 \(\mu\)g of 6-phosphofructo-2-kinase/fructose 2,6-bisphosphatase incubated with 280 micromolar of catalytic subunit; lane B4, autoradiogram of 6.25 \(\mu\)g of 6-phosphofructo-2-kinase/fructose 2,6-bisphosphatase incubated with 1.4 \(\mu\)g of rabbit skeletal muscle phosphorylase kinase. Lane C5, autoradiogram of 6.25 \(\mu\)g of glycogen phosphorylase b incubated with 1.4 \(\mu\)g of rabbit skeletal muscle phosphorylase kinase; lane C6, autoradiogram of 6.25 \(\mu\)g of glycogen phosphorylase b incubated with 1.4 \(\mu\)g of rabbit skeletal muscle phosphorylase kinase. Lane D7, autoradiogram of 6.25 \(\mu\)g of 6-phosphofructo-2-kinase/fructose 2,6-bisphosphatase incubated with 0.13 \(\mu\)g of rat liver phosphorylase kinase; lane D8, autoradiogram of 6.25 \(\mu\)g of glycogen phosphorylase b incubated with 0.13 \(\mu\)g of rat liver phosphorylase kinase.

\(^1\) In the zero time sample, there was a streaking of the nonphosphorylated form of the enzyme. This is frequently seen with purified enzyme preparations and may be due to charge modification of the protein of unknown origin. Some of the streaking may also represent phosphorylated forms of the enzyme since they all migrate as a single species after phosphorylation.

\(^2\) Garrison et al. (33, 34).

\(^3\) J. C. Garrison, personal communication.
phosphorylase kinase from rat liver and from rabbit skeletal muscle and the catalytic subunit of the cAMP-dependent protein kinase. In keeping with previous observations (11, 17, 23, 24), incubation of 6-phosphofructo-2-kinase/fructose 2,6-bisphosphatase (lane A1) with C subunit and [γ-32P]ATP and Mg2+ resulted in phosphorylation of 6-phosphofructo-2-kinase/fructose 2,6-bisphosphatase as visualized by autoradiography (lane B3); inclusion of the heat-stable inhibitor of C subunit completely prevented phosphorylation (lane B4). However, contrary to the report of Furuya et al. (17), skeletal muscle phosphorylase kinase (lane A2) was unable to phosphorylate 6-phosphofructo-2-kinase/fructose 2,6-bisphosphatase (lane C5) although it did, under the same conditions, phosphorylate glycogen phosphorylase b (lane C6). Also evident is the autophosphorylation of the α-subunit of phosphorylase kinase under these conditions (35, 36) with the β-subunit being phosphorylated to a lesser extent and not visible in this photograph. More important as regards the regulation of liver glycolysis, rat liver phosphorylase kinase was also unable to phosphorylate 6-phosphofructo-2-kinase/fructose 2,6-bisphosphatase (lane D7) under conditions where glycogen phosphorylase b was phosphorylated (lane D8). The conditions given in lane C5 were essentially the same as reported by Furuya et al. (17). Furthermore, alteration the incubation conditions (e.g., pH, protein kinase concentration, Ca2+ and calmodulin concentrations, Mg2+; incubation time) gave no indication that either liver or skeletal muscle phosphorylase kinase could phosphorylate 6-phosphofructo-2-kinase/fructose 2,6-bisphosphatase (data not shown). In addition, cyclic AMP-dependent phosphorylation resulted in reciprocal changes in 6-phosphofructo-2-kinase and fructose 2,6-bisphosphatase activities (23, 24) but incubation with phosphorylase kinase under any of the above conditions had no effect on enzyme activity (data not shown). We concluded from these studies that 6-phosphofructo-2-kinase/fructose 2,6-bisphosphatase was not a substrate for phosphorylase kinase.

Effect of Diabetes and Starvation on the Regulation of Hepatocyte Fructose 2,6-Bisphosphate Levels by Hormones and Glucose—The data presented in Figs. 2-4 show that fructose 2,6-bisphosphate levels can be regulated acutely by insulin as well as by glucagon and epinephrine. We have demonstrated that in diabetes the level of fructose 2,6-bisphosphate in freeze-clamped liver from fed rats is greatly reduced and that the total activity of 6-phosphofructo-2-kinase was reduced (22). It was of interest to study the effect of starvation and diabetes on the ability of glucagon, epinephrine, and phenylephrine and of glucose to regulate the level of fructose 2,6-bisphosphate (Table III). Incubation of hepatocytes from fed rats with 10 μM phenylephrine caused a slight increase in fructose 2,6-bisphosphate levels, presumably as a result of an increase in glycogenolysis (10). Epinephrine (10 μM) addition resulted in a decrease in the level of fructose 2,6-bisphosphate which was much less than that observed with glucagon. Addition of 30 mM glucose only raised fructose 2,6-bisphosphate levels slightly in hepatocytes from fed rats. The level of fructose 2,6-bisphosphate in hepatocytes from either starved or diabetic rats was greatly diminished compared to normal, fed rats. Addition of 30 mM glucose to hepatocytes from starved rats elevated the level of fructose 2,6-bisphosphate to near that of fed rats but had little or no effect on the level of fructose 2,6-bisphosphate in hepatocytes from diabetic rats. Glucagon, epinephrine, and phenylephrine had little or no effect in the starved or diabetic case. Addition of glucagon (10 nM) opposed the action of glucose to elevate fructose 2,6-bisphosphate levels in hepatocytes from 24-h starved rats. Addition of insulin alone to hepatocytes from fed, starved, and diabetic rats had no significant effect on the level of fructose 2,6-bisphosphate.

Effect of Starvation, Refeeding, and Diabetes on the Activity of Rat Liver 6-Phosphofructo-2-kinase—The results shown in Table III and those reported by us previously (22) suggest that the ability of glucagon and glucose to regulate the hepatic level of fructose 2,6-bisphosphate is dependent on the in vivo activity of 6-phosphofructo-2-kinase/fructose 2,6-bisphosphatase. This in turn depends on the total activity of the enzyme, its phosphorylation state, and the in vivo concentration of one of its substrate, fructose 6-phosphate, and of various effectors of the enzyme. Table IV shows that the total activity of the 6-phosphofructo-2-kinase is decreased in long term starvation and diabetes but little affected by 18 h of starvation. Refeeding rats starved for 72 h with a high carbohydrate diet for 48 h restored the level of the enzyme to greater than the normal fed case.

**Table III**

**Regulation of fructose 2,6-P2 levels by hormones and glucose in hepatocytes from fed, starved, and diabetic rats**

<table>
<thead>
<tr>
<th>Additions</th>
<th>Fructose 2,6-bisphosphate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Fed</td>
</tr>
<tr>
<td></td>
<td>mmol/g</td>
</tr>
<tr>
<td>None (6)</td>
<td>10.8 ± 1.2</td>
</tr>
<tr>
<td>Glucose, 30 mM (4)</td>
<td>15.1 ± 1.8</td>
</tr>
<tr>
<td>Glucagon, 10 mM (6)</td>
<td>0.7 ± 0.2</td>
</tr>
<tr>
<td>Glucose + glucagon (3)</td>
<td>0.9 ± 0.4</td>
</tr>
<tr>
<td>Epinephrine, 10 μM (4)</td>
<td>7.3 ± 1.0</td>
</tr>
<tr>
<td>Phenylephrine, 10 μM (3)</td>
<td>13.2 ± 0.7</td>
</tr>
</tbody>
</table>

**Table IV**

**Effect of starvation and diabetes on the activity of rat liver 6-phosphofructo-2-kinase**

Alloxan diabetes was induced by intravenous injection of alloxan monohydrate (60 mg/kg) in 0.9% saline; animals were used 48 h later if the blood glucose level was greater than 300 mg/100 ml. Animals were starved for 72 h and then refed a high carbohydrate diet for 48 h. Enzyme activities were measured with saturating concentrations of substrate as described under "Materials and Methods.

<table>
<thead>
<tr>
<th>Condition</th>
<th>6-Phosphofructo-2-kinase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal, fed (6)</td>
<td>3.6 ± 0.5</td>
</tr>
<tr>
<td>Starved, 18 h (3)</td>
<td>3.2 ± 0.4</td>
</tr>
<tr>
<td>Starved, 72 h (3)</td>
<td>0.9 ± 0.1</td>
</tr>
<tr>
<td>Starved, 72 h (3); refed, 48 h</td>
<td>4.5 ± 0.3</td>
</tr>
<tr>
<td>Diabetes (3)</td>
<td>1.2 ± 0.4</td>
</tr>
</tbody>
</table>

**Discussion**

Our results clearly demonstrate that insulin exerts acute effects on fructose 2,6-bisphosphate metabolism in rat liver. Insulin opposes the action of physiological concentrations of glucagon and high concentrations of catecholamine to lower the level of fructose 2,6-bisphosphate and the concentrations of insulin needed are also in the physiological range. Since the hepatic actions of glucagon are thought to be mediated by alterations in the level of cyclic AMP and insulin has been shown to oppose the action of glucagon to elevate cyclic AMP (19, 30), it seems reasonable to postulate that insulin modulates fructose 2,6-bisphosphate levels by opposing the cAMP-dependent phosphorylation of 6-phosphofructo-2-kinase/fructose 2,6-bisphosphatase. Consistent with a phosphorylation mechanism are the results in Table II.
where the effect of insulin on both 6-phosphofructo-2-kinase and fructose 2,6-bisphosphatase activity was observed in (NH₄)_2SO₄-heated extracts where the influence of low molecular weight effectors should be eliminated. Further evidence in support of this notion includes the ability of insulin to oppose the effect of exogenously added cAMP on fructose 2,6-bisphosphate levels (Fig. 3). While most of the evidence supports the hypothesis that glucagon lowers fructose 2,6-bisphosphate levels by a CAMP-dependent phosphorylation mechanism, there are several observations which are apparently not completely consistent with this hypothesis. First, concentrations of glucagon which have no measurable effect on the level of cyclic AMP (19) lower the level of fructose 2,6-bisphosphate by greater than 90% (Fig. 2). Second, insulin counteracts the effect of glucagon under conditions where both hormones have no measurable effect on cyclic AMP levels (Ref. 19; data not shown). These data suggest either that a cyclic AMP-independent mechanism may be involved or that small perhaps localized changes in cyclic AMP levels, beyond present limits of detection, may bring about change in 6-phosphofructo-2-kinase/fructose 2,6-bisphosphatase activity. There are a number of reasons for believing that the latter alternative is correct. First, 6-phosphofructo-2-kinase/fructose 2,6-bisphosphatase is an excellent substrate for the cyclic AMP-dependent protein kinase having a very low Km for the protein kinase (23). This probably accounts for the sensitivity of fructose 2,6-bisphosphate levels to glucagon. We have been able to detect small but statistically significant changes in the activity ratio of the CAMP-dependent protein kinase in extracts of cells incubated with 10⁻¹¹ to 10⁻¹⁰ M glucagon and Cherrington et al. (37) have also reported such changes in protein kinase activity with the same low concentrations of glucagon. Second, cyclic AMP-dependent phosphorylation of the enzyme results in changes in both the phosphotransferase and phosphohydrolase activities and this combination of effects may account in part for the sensitivity of fructose 2,6-bisphosphate levels to glucagon addition. Third, fructose 2,6-bisphosphate levels were more sensitive to the addition of exogenous cyclic AMP than were gluconeogenesis or pyruvate kinase activity (Table I), suggesting that the 6-phosphofructo-2-kinase/fructose 2,6-bisphosphatase is more sensitive to changes in intracellular cyclic AMP. Furthermore, we have been unable to demonstrate any cyclic AMP-independent decrease in the level of fructose 2,6-bisphosphate by employing various α-adrenergic agonists. Hue et al. (10) reported that α-adrenergic agonists and vasopressin actually elevated the level of fructose 2,6-bisphosphate in hepatocytes from fed rats and we have also observed such a direct effect of phenylephrine (Table IV). It is of course possible that insulin acts on the phosphatase which dephosphorylates 6-phosphofructo-2-kinase/fructose 2,6-bisphosphatase which would presumably result in a higher ratio of phosphotransferase to phosphohydrolase activity. Studies on the identification and characterization of such a phosphoprotein phosphatase are in progress.

The data presented in Fig. 2 and Table I suggest that fructose 2,6-bisphosphate levels were maximally altered at levels of glucagon where only minor changes in gluconeogenesis or pyruvate kinase activity were evident. Richards et al. (32) have reported similar results with hepatocytes and Nieto and Castano (38) found that administration of glucagon to intact rats also resulted in changes in 6-phosphofructo-1-kinase activity at lower concentrations of hormone than required for changes in pyruvate kinase activity. Thus, it would appear that fructose 2,6-bisphosphate levels are more sensitive to glucagon or cAMP than is pyruvate kinase or gluconeogenesis but the physiologic significance of this finding is not clear. Since fructose 1,6-bisphosphate is an inhibitor of the phosphorylation of pyruvate kinase (39), it might be expected that glucagon would first lower the level of fructose 1,6-bisphosphate, via alterations in fructose 2,6-bisphosphatase metabolism, prior to changes in pyruvate kinase and gluconeogenesis (38). However, it has not as yet been demonstrated that glucagon-induced reduction in fructose bisphosphate levels precedes changes in gluconeogenesis, although this is still a viable hypothesis (38).

Our interpretation of the effects of catecholamines in rat liver is somewhat different from that of Uyeda and co-workers (18, 32) who claim that epinephrine lowers fructose 2,6-bisphosphate and causes a large inhibition of 6-phosphofructo-2-kinase activity and activation of fructose 2,6-bisphosphatase activity (18) by a mechanism involving activation of phosphorylase kinase (17). These effects of epinephrine were only observed when cells were incubated with calcium and were not obtained in hepatocytes depleted of their calcium stores by treatment with ethylene glycol bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid (15). These results are puzzling since it is well known that hepatocytes that have been depleted of Ca²⁺ show a paradoxical increase in the level of cyclic AMP in response to β-adrenergic agonists (40). We find that epinephrine causes a decrease in fructose 2,6-bisphosphate in Ca²⁺-depleted cells (data not shown). Richards et al. (18) also stated that Hue et al. (10) had observed a phenylephrine-induced decrease in the level of cyclic AMP. However, Hue et al. (10) actually reported that phenylephrine, both in the presence and absence of β-blocker, elevated the level of fructose 2,6-bisphosphate. Hue et al. (10) did report that phenylephrine and vasopressin did not elevate fructose 2,6-bisphosphate levels in hepatocytes which had been treated with ethylene glycol bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid and that isoproterenol, a β-agonist, lowered the level of the compound. Hue et al. (10) argued that the α-adrenergic-induced increase in fructose 2,6-bisphosphate levels resulted from the provision of fructose 6-phosphate as a result of enhanced glycogenolysis and not from changes in 6-phosphofructo-2-kinase activity. We have observed an epinephrine-induced decrease in fructose 2,6-bisphosphatase levels and a corresponding alteration in the activities of 6-phosphofructo-2-kinase/fructose 2,6-bisphosphatase but the effect on fructose 2,6-bisphosphate levels was smaller than that for glucagon and was not seen in the presence of a β-blocker. Thus, it appears that the β-adrenergic component of the catecholamines induced a decrease in fructose 2,6-bisphosphate levels by a CAMP-dependent alteration in the phosphorylation state of 6-phosphofructo-2-kinase/fructose 2,6-bisphosphatase. This effect is small and slow in onset compared to glucagon and these results correlate well with the small effect of epinephrine on cyclic AMP levels in rat liver compared to glucagon (20). The effect of α-adrenergic agonists in the rat liver system is less certain since these agents have been reported to elevate (Table IV; Ref. 10), decrease (18), or to have no effect on the levels of fructose 2,6-bisphosphate (data not shown). The discrepancy between the various reports may be due to differences in hepatocyte preparation and accompanying differences in glycogen and calcium stores.

We were unable to demonstrate phosphorylation of 6-phosphofructo-2-kinase/fructose 2,6-bisphosphatase catalyzed by either skeletal muscle or purified rat liver phosphorylase kinase and no changes were observed in kinetic properties

\(^{1}\) S. J. Pilkis, unpublished results.

upon incubation of the enzyme with phosphorylase kinase in the absence or presence of calcium and calmodulin (data not shown). In addition, calcium-linked hormones, presumably acting via phosphorylation mechanisms, have no effect on the phosphorylation state of the enzyme in intact hepatocytes (33, 34). These results are in contrast to the report of Furuya et al. (17) who claimed that incubation of the enzyme with partially purified phosphorylase kinase from skeletal muscle in the presence of calcium and calmodulin resulted in inhibition of 6-phosphofructo-2-kinase activity. However, they did not directly demonstrate $^{32}$P incorporation into 6-phosphofructo-2-kinase and their incubations were done in the absence of protein kinase inhibitor. Therefore, it is possible that the activity change was due to the presence of the catalytic subunit of cAMP-dependent protein kinase or other protein kinases in their phosphorylase kinase or 6-phosphofructo-2-kinase preparations. Our finding that rat liver phosphorylase kinase did not catalyze phosphorylation of the purified enzyme casts doubt on this as a physiologically relevant control mechanism. It is still possible that the enzyme may be regulated via a cAMP-independent phosphorylation mechanism which does not involve phosphorylase kinase. Attempts to catalyze the phosphorylation of the 6-phosphofructo-2-kinase/fructose 2,6-bisphosphatase with the calmodulin-sensitive glycogen synthase kinase from rat liver described by Payne and Sodering (41) were unsuccessful (data not shown).

In addition to acute regulation of the level of fructose 2,6-bisphosphate by hormones, the metabolism of the compound is greatly altered in diabetes. The low level of fructose 2,6-bisphosphate observed in diabetes is probably a result of both decreased glucokinase activity (22) and 6-phosphofructo-2-kinase activity (22). Glucose addition to hepatocytes from diabetic animals did not elevate fructose 2,6-bisphosphate levels whereas it did elevate the level of the compound in hepatocytes from rats starved for 24 h. Neither glucokinase (22) nor 6-phosphofructo-2-kinase activity (Table I) is significantly reduced after only 24 h of starvation. Thus, after 24 h of starvation, glucose-phosphorylating capacity would not be reduced and addition of 30 mm glucose would provide fructose 6-phosphate for conversion to fructose 2,6-bisphosphate. Decreased glucokinase activity in the diabetic case would limit glucose phosphorylation and, thus, the concentration of fructose 6-phosphate available as substrate for 6-phosphofructo-2-kinase. Cyclic AMP levels are also elevated in livers from diabetic rats (39) and it is likely that 6-phosphofructo-2-kinase/fructose 2,6-bisphosphatase is in the phosphorylated form where phosphotransferase activity is inhibited and phosphorylase activity is activated. In addition, synthesis of fructose 2,6-bisphosphate in the diabetic state is also probably limited by the decreased total activity of 6-phosphofructo-2-kinase. These factors probably contribute to the greatly diminished rate of glycolysis and enhanced rate of gluconeogenesis that is observed in the diabetic liver (42).

In summary, the results presented here show that insulin exerts minute-to-minute regulation of fructose 2,6-bisphosphate metabolism via alterations in the phosphorylation state of 6-phosphofructo-2-kinase/fructose 2,6-bisphosphatase and that the hormone may also modulate the amount of the enzyme present in liver. Since fructose 2,6-bisphosphate is the most important physiological regulator of 6-phosphofructo-1-kinase and fructose 1,6-bisphosphatase (1) and *ipsa facto* important in the regulation of hepatic glycolysis and gluconeogenesis, it is likely that effects of insulin on the level of the compound are significant in the hormone’s modulation of hepatic carbohydrate metabolism.

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