Role of Enzyme-Enzyme Interactions in the Regulation of Glycolysis

INACTIVATION OF D-FRUCTOSE 1,6-DIPHOSPHATASE BY KIDNEY CORTEX MITOCHONDRIA*

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JOSEPH MENDICINO, H. S. PRIHAR, AND FATHI M. SALAMA

From the Department of Biochemistry, Ohio State University, Columbus, Ohio 43210

SUMMARY

Particulate preparations obtained from a number of tissues, including liver, kidney, and muscle inactivated renal D-fructose 1,6-diphosphatase when they were incubated with the enzyme in the presence of adenosine triphosphate, magnesium ion, and cysteine. Purified swine kidney D-fructose 1,6-diphosphatase and rat kidney uridine diphosphate-D-glucose-glycogen glucosyltransferase were inactivated by washed rat kidney cortex mitochondria. The initial rate of the reaction was dependent on the concentration of mitochondria, D-fructose 1,6-diphosphatase, and adenosine nucleotides, and magnesium ion and cysteine were required for maximum activity. The rate of inactivation of the enzyme by mitochondria was stimulated by the addition of adenosine diphosphate and adenosine monophosphate and to a lesser extent by adenosine triphosphate. The addition of malate and succinate to the incubation mixture decreased the rate of inactivation. Under the experimental conditions used in this study the rate of inactivation was approximately $1.1 \times 10^{-3}$ pmole of D-fructose 1,6-diphosphatase per min per g of tissue.

The synthesis of glycogen and glucose from noncarbohydrate precursors such as lactate, pyruvate, and amino acids is known to occur in kidney cortex. Present evidence suggests that the rate of glycolysis and gluconeogenesis in this tissue may be controlled at the glycogen and D-fructose 1,6-diphosphate levels (1-3). The results of many studies indicating that the activity of D-fructose 1,6-diphosphatase was varied by diet and the administration of hormones encouraged us to examine the characteristics of this enzyme in kidney.

D-Fructose 1,6-diphosphatase was isolated from swine kidney extracts and highly purified preparations with specific activities of 25 pmoles per min per mg of protein have been obtained (4, 5). The enzyme was inhibited by AMP and high concentrations of D-fructose 1,6-diphosphate (5). The nature of the regulatory effects of these compounds on the activity of the enzyme has been examined in several tissues. The kinetic properties of specific D-fructose 1,6-diphosphatases and the allosteric inhibition by AMP have been reported for enzymes purified from rat liver (6, 7), bovine liver (8), Candida utilis (9), and Escherichia coli (10). The inhibition by AMP is noncompetitive with respect to D-fructose 1,6-diphosphate and is reversed upon removal of AMP. The enzyme can also be dissociated into subunits under a variety of conditions (6, 11-13). These results indicate that the enzyme in the presence of AMP, D-fructose 1,6-diphosphate, or sulfhydryl reagents can undergo reversible changes in its tertiary and quaternary structure which affect its catalytic and allosteric properties (14, 15).

Another more specific and direct mechanism for the frequently proposed involvement of D-fructose 1,6-diphosphatase in the regulation of the rate of gluconeogenesis was suggested by the finding that the purified kidney enzyme was inactivated in the presence of crude kidney extracts, ATP, and magnesium ion (16). These studies further indicated that epinephrine and other hormonal factors might exert their regulatory effects on carbohydrate metabolism by simultaneously stimulating and inactivating appropriate reactions involved in the multienzyme systems which catalyze the degradation and synthesis of glucose and glycogen in liver and kidney (16). Protein kinases and phosphoprotein phosphatases present in extracts of various animal tissues have been shown to influence the activity of phosphorylase (2, 3, 17) and UDP-D-glucose-glycogen glucosyltransferase (18, 19). Similar enzymes may also be involved in the regulation of the activity of D-fructose 1,6-diphosphatase and phosphofructokinase.

The present communication reports in more detail on the nature of the system which inactivates D-fructose 1,6-diphosphatase in kidney cortex extracts. The inactivating system was found to be located in the mitochondria and the properties of this particular system were examined. The possible physiological relationship of this system to the regulation of glycolysis and gluconeogenesis is discussed.
EXPERIMENTAL PROCEDURE

Purification of D-Fructose 1,6-Diphosphatase from Swine Kidney—In order to use the enzyme as a substrate in the present studies it was necessary to prepare large quantities of purified D-fructose 1,6-diphosphatase and to develop methods which would permit the recovery of unreacted enzyme from incubation mixtures. The enzyme was purified from swine kidney by ammonium sulfate precipitation and chromatography on cellulose-P as described previously (4, 5), except that after elution from the first large cellulose-P column (12 x 18 cm) it was always kept and stored in the presence of 0.01 M β-mercaptoethanol and 0.0004 M 8-hydroxyquinoline. The latter compound greatly increased the stability of the purified enzyme.

Since the crude extracts contained enzymes capable of altering the activity of D-fructose 1,6-diphosphatase it was necessary to obtain preparations which were virtually free of these systems before an examination of the mechanism could be safely carried out. Furthermore, since only a small amount of the added D-fructose 1,6-diphosphatase was utilized in the inactivation mixtures, the purification procedure was modified to permit the recovery of this enzyme. All solutions containing active enzyme were diluted to 0.1 M NaCl and added to the first cellulose-P eluent before the concentration step on small cellulose-P columns (5 x 10 cm) (4). After precipitation with ammonium sulfate (Fraction 3) D-fructose 1,6-diphosphatase was completely free of interfering enzymes.

Assay of Inactivating System—The assay was based upon the ability of the enzyme preparation to catalyze the inactivation of D-fructose 1,6-diphosphatase or UDP-D-glucose-glycogen glucosyltransferase when incubated with ATP in the presence of magnesium ion and cysteine (16). The system used for most of the experiments reported was incubated at 37° and in the routine assay contained, in 1 ml, 30 mM Tris-HCl (pH 8.0), 10 mM MgCl₂, 10 mM cysteine, 250 mM sucrose, 10 mM ATP, 4 to 10 μM D-fructose 1,6-diphosphatase, and an appropriate amount of the particulate enzyme. The reaction was started with D-fructose 1,6-diphosphatase concentrations well below those required to saturate the inactivating system in order to preserve enzyme. The reaction kinetics were zero order only for small conversions of substrate to product. When more than 15% conversion took place the reaction rates were calculated from the slopes of the initial linear portions of plots of the amount of decrease in D-fructose 1,6-diphosphatase activity. The reaction was stopped by immediately diluting and chilling the incubation mixture with 50 ml of 0.01 M Tris-HCl, pH 8.0, or 3°. The solution was then passed through a cellulose-P column and fructose 1,6-diphosphatase was quantitatively eluted from the column and assayed as described previously (16). A unit of activity in all cases represents the inactivation of 1 μmole of D-fructose 1,6-diphosphatase per min. (25 μmoles per min per mg × 130 mg per μmole); 3950 μmoles per min of activity represent 1 μmole of D-fructose 1,6-diphosphatase (4).

Assay of D-Fructose 1,6-Diphosphatase Activity—The standard procedure was based on the determination of P₁ formed from D-fructose 1,6-diphosphate at low substrate concentrations. The reaction mixture was incubated at 38° for 5 min and contained in 9 ml 100 mM Tris-HCl (pH 8.0), 12 mM MgCl₂, 5 mM cysteine, 0.08 mM D-fructose 1,6-diphosphate, and an appropriate amount of enzyme. One unit of activity is defined as the formation of 1 μmole of P₁ from fructose 1,6-di-P per min, and specific activity is expressed as units per mg of protein.

### TABLE I

<table>
<thead>
<tr>
<th>System</th>
<th>Crude extract</th>
<th>Purified D-fructose 1,6-diphosphatase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complete</td>
<td>0.85</td>
<td>1.53</td>
</tr>
<tr>
<td>Omit MgCl₂</td>
<td>1.38</td>
<td>1.48</td>
</tr>
<tr>
<td>Omit ATP</td>
<td>1.82</td>
<td>1.53</td>
</tr>
<tr>
<td>Omit ATP, MgCl₂</td>
<td>1.85</td>
<td>1.49</td>
</tr>
<tr>
<td>Omit ATP, add 1 mg of epinephrine</td>
<td>1.88</td>
<td>1.51</td>
</tr>
</tbody>
</table>

**Assay of UDP-D-glucose-glycogen glucosyltransferase**—The enzyme used in the assay was routinely prepared in the presence of 60 mM β-mercaptoethanol and 0.5% glycogen. The standard reaction mixture was incubated at 30° for 30 min and contained, in 0.3 ml, 50 mM Tris-HCl (pH 7.5), 10 mM cysteine, 6 mM UDP-D-glucose-³⁴C (40,000 cpm per μmole), 10 mM glucose-6-P, 4 mg of glycogen, and an appropriate amount of enzyme. The reaction was stopped by the addition of 2 ml of ethanol and the radioactivity incorporated into glycogen was measured as described previously (20). UDP-D-glucose-³⁴C was prepared from UTP and ³⁴C-glucose-6-P with the appropriate purified enzymes (20). All assays were carried out in the presence and absence of 0.01 M glucose-6-P.

**Preparation and Procedures**—The subcellular fractions used in distribution studies were prepared from fresh swine tissue or rat kidney according to the procedure described by Hogeboom (21) and Appelmans, Wattiaux, and de Duve (22). The particulate fraction used in these studies was selected on the basis of its ability to inactivate D-fructose 1,6-diphosphatase. Rat kidney mitochondria were the most convenient source of the inactivating system and they were isolated as follows: fresh kidneys were homogenized in 9 volumes of cold 0.25 M sucrose and the precipitate which sedimented between 600 x g and 5000 x g in 10 min was collected and washed twice by suspending it in the original volume of 0.25 M sucrose and centrifuging at 5000 x g. The concentration of mitochondria was determined by protein assay or by drying at 100° to a constant weight. Protein was estimated colorimetrically (23) and at low concentrations spectrophotometrically (24). Inorganic phosphate was assayed by the method of Fiske and SubbaRow (25).

**RESULTS**

**Influence of ATP and MgCl₂ on Activity of D-Fructose 1,6-diphosphatase in Crude Extracts and Purified Preparations**—The data presented in Table I clearly indicate that the addition of ATP and MgCl₂ to crude kidney extracts increased the rate of inactivation of D-fructose 1,6-diphosphatase. However, these compounds had no effect on the purified enzyme isolated from...
TABLE II

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Fraction</th>
<th>μmol/min/g x 10⁴</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 to 500 x g</td>
<td>500 x g to 3000 x g</td>
</tr>
<tr>
<td>Muscle</td>
<td>17</td>
<td>0</td>
</tr>
<tr>
<td>Brain</td>
<td>0</td>
<td>22</td>
</tr>
<tr>
<td>Liver</td>
<td>20</td>
<td>13</td>
</tr>
<tr>
<td>Kidney</td>
<td>4.8</td>
<td>9.6</td>
</tr>
</tbody>
</table>

these extracts. Epinephrine was inactive with both preparations. The turbid extracts used in these experiments were prepared by centrifugation of a 50% homogenate at 30,000 x g for 10 min. However, when the homogenate was centrifuged at 9,000 x g the activity of the extract was greater than after centrifugation at 30,000 x g. In an attempt to resolve this difficulty all of the particulate material in the preparation was removed by diluting the extract 10-fold and centrifuging it at 80,000 x g for 30 min. The clear supernatant solution was now inactive and almost all of the inactivating activity was found to be associated with the insoluble pellet.

**Distribution of Inactivating Enzyme in Swine Tissues**—The subcellular distribution of the inactivating enzyme in various tissues of the swine was examined (Table II). In muscle the activity was in the 0 to 500 x g, 10-min fraction, and in brain it was mainly in the 500 x g to 3000 x g, 10-min fraction. In the liver the activity was in the 0 to 3000 x g, 10-min fractions. The 500 x g to 3000 x g, 10-min fraction contained most of the activity in the kidney. In each case the enzyme was associated mainly with particulate material. Mitochondria or sarcosomes contained the major portion of the inactivating activity, and the microsomes and soluble fraction were practically devoid of activity. In muscle, kidney, and liver large amounts of activity were recovered in the 0 to 500 x g, 10-min fraction which contains nuclei, cell membranes, and whole cells. When these fractions from liver and kidney were homogenized again, appreciable amounts of inactivating activity were recovered in the 500 x g to 3000 x g, 10-min fractions. Thus, it appeared that the enzyme inactivating d-fructose 1,6-diphosphatase was located principally in the light and heavy mitochondria fractions. With the use of the more active fractions, the influence of other factors was then examined.

**Effect of Treatment with Deoxycholate, Triton X 100, and Tween 80 upon Particulate-inactivating System**—The inactivating system is very tightly bound to the mitochondria prepared from swine kidney, since it could not be removed by washing with 0.154 M KCl, 0.5 M Tris-HCl, pH 8.0, or with detergents.

One volume of a suspension of the 500 x g to 34,000 x g particulates, derived from 10 g of swine kidney, was treated at 3° and pH 7.5 with an equal volume of 0.5% and 1% sodium deoxycholate, Triton X-100, or Tween 80. The mixture was stirred for 20 to 30 min and then centrifuged at 80,000 x g for 60 min. The precipitate and supernatant were then assayed for d-fructose 1,6-diphosphatase-inactivating activity. This treatment removed most of the water-soluble protein from the mitochondria, but the inactivating enzyme was still present in the particulate fraction, in each case. After treatment with 0.5% solutions of deoxycholate, Tween 80, or Triton X-100 the particles contained 0.8, 0.6, and 1.15 x 10^{-4} unit of activity compared to 0.05, 0.05, and 0.08 x 10^{-4} unit of activity in the supernatant fractions. The amount of activity solubilized was negligible and the total activity recovered after treatment with these detergents usually decreased. The untreated particles used in these experiments contained 1.17 x 10^{-4} unit of activity. Several other procedures including extraction with 5% butanol or 20% ethanol at -20° also resulted in an active precipitate and an inactive supernatant. The enzyme was released very slowly when the preparation was subjected to sonic disintegration. Many of the enzymes loosely bound to the mitochondria can be removed by disruption of these particles; however, those attached to the membrane would remain insoluble. Since it is unlikely that d-fructose 1,6-diphosphatase enters the intact particle to be acted on, it would appear that the inactivating system is attached to the membrane of the mitochondria.

The inactivating enzyme remained bound to the mitochondria during the assay. Centrifugation of the standard reaction mixture at 80,000 x g for 5 min essentially stopped the reaction in the supernatant, whereas the reaction was resumed when the pellet containing mitochondria was resuspended in fresh incubation mixture. Since drastic treatment of the mitochondrial membranes and rigorous washing of the mitochondria during isolation failed to solubilize the d-fructose 1,6-diphosphatase-inactivating activity to any significant extent, it may be concluded that this activity is due to a particulate enzyme sedimenting with mitochondria and not to a contamination of mitochondria with a soluble enzyme.

**Requirements of Reaction**—The requirements for the inactivation of purified d-fructose 1,6-diphosphatase by rat kidney mitochondria are shown in Table III. The activity of the inactivation system was completely dependent on the presence of mitochondria. Some activity was observed in the absence of added ATP, MgCl₂ and cysteine since they are endogenous constituents of the mitochondria. The omission of malate and succinate from the incubation mixture increased the rate of the reaction compared to the complete system (3.16 compared to 2.38). This unexpected finding further indicates that the inactivating system is present in the mitochondria, and indeed that it may be regulated by other reactions occurring in this subcellular compartment.

**Specificity of Reaction and Inactivation of UDP-d-glycogen Glucosyltransferase**—The kidney does not store large quantities of glycogen and the major product of gluconeogenesis in this tissue is glucose. However, the synthesis of some glycogen from non-
carbohydrate precursors such as lactate, pyruvate, and amino acids does occur in kidney cortex. The activity of UDP-D-glucose-glycogen glucosyltransferase in the 80,000 g, 30-min supernatant fraction prepared from rat kidney was 22 to 31.9 μmoles per hour per g, wet weight, of tissue under the standard assay conditions.

The possible relationship of this reaction, which is also involved in the synthesis of glycogen during glyconeogenesis, to the inactivation of fructose 1,6-diphosphatase was studied by examining the specificity of the mitochondrial system with respect to the inactivation of this enzyme. The requirements for the inactivation of the form of UDP-D-glucose-glycogen glucosyltransferase dependent on glucose-6-P were similar to those of fructose 1,6-diphosphatase as shown in Table IV, whereas the activity in the presence of glucose-6-P was only slightly decreased on incubation with mitochondria. The activity relative to the complete system was calculated on the basis of the amount remaining after incubation in each case. These results suggest that the same mitochondrial system is responsible for the inactivation of both of these enzymes.

Relation of Mitochondria Concentration to Reaction Velocity—The activity of the inactivating system was linear with time and proportional to the concentration of mitochondria only for very short incubation times (Fig. 1). The control used in these experiments was a complete reaction mixture with heated mitochondria, since the activity of this sample was not significantly altered on incubation at 37°. Under the conditions indicated in the figure, in 2 min 3 mg, 6 mg, and 9 mg of mitochondria inactivated 1.6 × 10⁻⁴, 3 × 10⁻⁴, and 4.4 × 10⁻⁴ μmole of fructose 1,6-diphosphatase, respectively. The decreased rate of the reaction after 2 min was due to the utilization of the substrate and the hydrolysis of ATP, especially at high concentrations of mitochondria. With the use of the approximate physiological concentration of mitochondria in swine kidney in the incubation mixtures, a half-time for the inactivation of fructose 1,6-diphosphatase of less than 1 min was observed.

Influence of Fructose 1,6-Diphosphatase Concentration on Rate of Reaction—The effect of fructose 1,6-diphosphatase concentration on the initial rate of the inactivating reaction is shown in Fig. 2. Concentration on the initial rate of the inactivating reaction is shown in Fig. 2. Since both the catalyst and the substrate are proteins one can no longer assume pseudo first-order kinetics as enzyme and substrate may be present in about equimolar amounts. Moreover, since the inactivating system is bound to a particle its effective concentration cannot be determined. The rate of the reaction was completely dependent on substrate up to a concentration of approximately 3 × 10⁻⁴ M. The quantity of enzyme (0.03 μmole per ml) in this incubation mixture is approximately...
A mixture was incubated for 10 min at 37°C and contained, in 1 ml, 30 mM Tris-HCl (pH 7.0), 10 mM MgCl₂, 20 mM ATP, 15 μM cysteine, the concentration of α-fructose 1,6-diphosphatase indicated in the figure, and approximately 3 mg dry weight, of mitochondria.

The concentration of inactive α-fructose 1,6-diphosphatase in resting muscle extracts was found to contain mainly phosphorylase b rather than phosphorylase a. Since resting muscle contains relatively high steady state levels of ATP and very little AMP the activity of the phosphorylase reaction would be very low. If glycogen synthetase was active under these conditions the synthesis of glycogen would be favored in resting muscle. It was of interest then to determine whether α-fructose 1,6-diphosphatase was in an active or inactive form in kidney cortex tissue used in the present studies.

Exactly 50 g of tissue from the cortex of 10 kidneys obtained from different animals were homogenized in 100 ml of 0.05 M Tris-HCl, pH 8.0, and centrifuged at 34,000 × g for 10 min. The precipitate was washed once with another 50 ml of 0.05 M Tris-HCl, pH 8.0. The combined extracts were diluted to 200 ml, a 2-ml aliquot in each case was applied to a cellulose-P column, and α-fructose 1,6-diphosphatase was isolated and assayed as described under "Experimental Procedure." The activities in micromoles per min per g of tissue from 10 different kidneys were 11.0, 9.1, 5.8, 10.5, 10.8, 10.7, 16.5, 14.5, 25.3, and 10.8. In these experiments aliquots of the extracts at 3°C were immediately diluted with 50 ml of 0.01 M Tris-HCl, pH 8.0, at 3°C and applied to cellulose-P columns. When additional samples were removed from the extracts at 2 and 4 hours, the same activities were obtained, indicating that no change in the activity of the enzyme had occurred in the extracts on standing at 3°C. Thus, on the average, the activity of α-fructose 1,6-diphosphatase in swine kidney was approximately 10 μmol per min per g of tissue.

The concentration of inactive α-fructose 1,6-diphosphatase was measured by incubating a dialyzed sample of each extract in the presence of 0.01 M MgCl₂ for 5 to 15 min at 37°C as described previously (16). No significant increase in the activity of α-fructose 1,6-diphosphatase was observed in any of the extracts. However, when purified inactive α-fructose 1,6-diphosphatase prepared by incubation with the inactivating system was added to the dialyzed extracts the enzyme was completely reactivated, which indicated that an activating system was present in the extract (16).

The possibility that the observed effects occurred during homogenization or after centrifugation and removal of the particulate-inactivating system was examined by homogenizing fresh kidney cortex tissue in buffer containing 0.1 M sodium fluoride, 0.001 M 8-hydroxyquinoline, or 0.002 M Versene. These compounds would be expected to inhibit the activating and inactivating systems present in the extracts, since both require divalent metal ion. The addition of these compounds to the buffer had no significant influence on the total activity or form of α-fructose 1,6-diphosphatase in swine kidney. Moreover, when fresh swine kidney cortex tissue was homogenized in buffer containing inactive α-fructose 1,6-diphosphatase the same quantity of active α-fructose 1,6-diphosphatase was found as in a control sample homogenized in buffer only. These results indicate that although the content of α-fructose 1,6-diphosphatase in kidney extracts varies over a relatively wide range (8.5 to 25.3 units per g) the enzyme is present mainly in an active form. This result would indicate that the resting tissue is normally poised for gluconeogenesis.

The precipitates obtained by centrifugation of the same swine
kidney homogenates at 34,000 × g were washed twice with 50 ml of 0.05 M Tris-HCl, pH 8.0, and then were assayed for inactivating activity. In each case, the reaction mixture was incubated for 10 min at 37° and contained, in 1 ml, 30 mm Tris-HCl (pH 8.0), 10 mm cysteine, 10 mm MgCl₂, 20 mm ATP, and 0.01 mm D-fructose 1,6-diphosphatase. The rates of inactivation of D-fructose 1,6-diphosphatase in nanomoles of enzyme per min per g of tissue from seven kidneys were 1.01, 1.05, 1.01, 1.05, 0.64, 1.07, and 1.10. One gram of renal tissue contains approximately 10 μmoles per min per g of D-fructose 1,6-diphosphatase activity which is equivalent to about 0.0031 μmol of D-fructose 1,6-diphosphatase per g of renal tissue (10 μmoles per min per g divided by 3250 μmoles per min of activity per micromole of enzyme protein). Under the conditions of the assay system used in these studies, the inactivating system present in 1 g of tissue could convert all of the D-fructose 1,6-diphosphatase present to an inactive form in about 3 min (0.0031 μmol of enzyme per g divided by 0.001 μmol inactivated per min per g).

This calculation can be considered only a first approximation, since other factors such as the concentration of mitochondria and the decreasing rate of the reaction resulting from a decreasing concentration of substrate as well as numerous other variables have not been taken into consideration. However, it does indicate that this type of metabolic control may be more direct, specific, and immediate than those which are accompanied by an increase or reduction in the amount of enzyme being synthesized in the cell.

**Discussion**

The results obtained in these studies support and extend previous observations which showed that ATP and magnesium ion suppressed the activity of D-fructose 1,6-diphosphatase in crude kidney extract (16), and also provide additional evidence for the increasing recognition of an integrated relationship between the simultaneous activation of phosphofructokinase and phosphorylase and the inactivation of D-fructose 1,6-diphosphatase and UDP-D-glucose-glycogen glucosyltransferase accompanying the stimulation of glycolysis. On the basis of evidence presently available, a current working hypothesis, shown in Fig. 4, was formulated which depicts some of the possible interrelationships between these enzymes during glycolysis and gluconeogenesis. The activation of an enzyme system present in the mitochondria, which is sensitive to both the level of AMP and ADP (Fig. 3) in the soluble fraction of the cell and to the influence of hormones outside the cell, can very rapidly reverse the direction of carbohydrate metabolism from gluconeogenesis to glycolysis. Epinephrine may not have to be taken up by the cell to be effective, since it probably interacts with an enzyme such as adenylyl cyclase in the peripheral structure of the cell wall causing an alteration in the concentration of a cellular constituent such as cyclic 3',5'-AMP (3). This compound may stimulate the mitochondrial protein kinase which in turn catalyzes a glycolytic response.

The gluconeogenic state would be restored after the compounds produced by high blood levels of the hormone were destroyed or stored, or when the AMP and ADP in the soluble cytoplasmic fraction of the cell had been phosphorylated to ATP (Fig. 3). Thus, when the concentration of epinephrine decreased in the extracellular fluid, the formation of cyclic 3',5'-AMP would decrease and the cyclic nucleotide already present in the cell would be hydrolyzed by a specific phosphatase (3). The mitochondrial protein kinase might then be unable to suppress the activity of the soluble phosphoprotein phosphatase. If the phosphoprotein phosphatase were thus free to act it would dephosphorylate the phosphoenzymes in the soluble fraction of the cytoplasm and slowly restore a glyconeogenic state, as shown in Fig. 4. Since the enzyme would also have to dephosphorylate its inactivated form in this scheme the recovery from glycolysis would be slow and somewhat autocatalytic. In kidney the major product of gluconeogenesis is glucose; the enzyme glucose 6-phosphatase might also be inactivated and reactivated in this tissue.

The physiological parameters obtained by studies in vivo are compatible with this proposal. Many physiological and pharmacological conditions which activate phosphorylase lead to a rapid disappearance of glycogen, whereas, the rate of reactivation is much slower (28, 29). The process which stimulates glycolysis under the influence of epinephrine or other factors is very active and has a very rapid response. Thus, for example, recently Lowry et al. (30) found that when the blood supply to the brain of anesthetized immature mice was suddenly cut off there was a 6-fold increase in the glycolytic rate within a few seconds. The increased activity was associated with decreases in the tissue levels of glucose 6-P and fructose 6-P and increases in the level of D-fructose 1,6-diP and all the intermediates between D-fructose 1,6-diP and lactate. These results indicated that the phosphorylation of fructose 6-P was greatly facilitated in the first few seconds. The results did not distinguish between enzyme activation and inactivation or the multitude of other mechanisms which could explain these effects. However, an activation of the system described in this report would be consistent with this type of observation. Collectively these reactions, closely intertwined with one another, may express the characteristic glycolytic effects which epinephrine elicits when it acts on the kidney and liver cell. The separate but similar action of the protein kinase present in the mitochondria on specific enzymes present in the soluble fraction of the cytoplasm may, in turn, help to explain the peculiar ability of one organic compound to alter both the direction and the rate of an entire system of interrelated catalysts.

The induction of high rates of gluconeogenesis by diabetes, starvation, and treatment with steroids are associated with the release of large amounts of free fatty acids (31). Moreover, the synthesis of glucose from lactate and amino acids in tissue aldes

![Fig. 3. The influence of adenosine nucleotide concentration on the rate of inactivation of D-fructose 1,6-diphosphatase. The reaction was measured by the standard assay procedure. The reaction mixture was incubated at 37° for 5 min and contained, in 1 ml, 30 mm Tris-HCl (pH 7.5), 250 mm sucrose, 10 mm cysteine, 10 mm MgCl₂, 5 mm succinate, 5 mm malate, 10 mm Pi, 8 mm D-fructose 1,6-diphosphatase, 2 mg, dry weight, of mitochondria, and the concentration of each adenosine nucleotide indicated in the figure.](http://www.jbc.org/)

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from kidney and liver is stimulated by the addition of free fatty acids (31, 32). This stimulation of gluconeogenesis by dietary fat and high levels of fatty acids (33) is consistent with the proposed mechanism. The oxidation of fatty acids by the mitochondria would be expected to maintain a high ratio of ATP to ADP in the soluble cytoplasmic fraction which would directly aid gluconeogenesis, and at the same time this high ratio would indirectly suppress the mitochondrial system which inactivates d-fructose 1,6-diphosphatase and glycogen synthetase, thus helping to maintain a gluconeogenic state in the soluble fraction. At low concentrations of adenosine nucleotides there is practically no activity with added ATP as shown in Fig. 3. In the absence of fatty acids the regulatory system present in the mitochondria would still be responsive to the energy needs of the cell, only in this case, as the levels of ADP and AMP in the soluble fraction of the cytoplasm increase, the mitochondrial system would catalyze a glycolytic response. This mechanism is different from the often proposed direct influence of the ATP to ADP ratio on the activity of the glycolytic reactions themselves, since in this case variations in the ratio are acting as a signal to an enzyme system present in the mitochondria which in turn regulates the activity of glycolytic enzymes in the soluble fraction of the cytoplasm.

There is no evidence as yet for the phosphorylation and dephosphorylation of d-fructose 1,6-diphosphatase. The enzyme system detected in mitochondria which catalyzes the inactivation of d-fructose 1,6-diphosphatase and UDP-d-glucose-glycogen glucosyl-transferase does not appear to be directly related to any of the other protein kinases described previously (1-3). Only the other kinases and phosphoprotein phosphatases acting on liver, heart and muscle phosphorylase have been examined extensively (1, 2), and this is the only enzyme in Fig. 4 that has been unequivocally shown to undergo phosphorylation and dephosphorylation reactions. The liver phosphoprotein phosphatase acted on several other phosphoproteins besides phosphofructokinase, but it did not dephosphorylate non-protein phosphate esters (34, 35). The phosphorylase kinase on the other hand appears to be more specific (1). Recent studies have shown that rat liver mitochondria can phosphorylate phosvitin and dephosphorylate (36). The possible relationship of this system to the one described in this report remains to be examined.

REFERENCES
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