A reversible interconversion of two kinetically distinct forms of hepatic pyruvate kinase regulated by glucagon and insulin is demonstrated in the perfused rat liver. The regulation does not involve the total enzyme content of the liver, but rather results in a modulation of the substrate dependence. The forms of pyruvate kinase in liver homogenates are distinguished by measurements of the ratio of the enzyme activity at a subsaturating concentration of P-enolpyruvate (1.3 mM) to the activity at a saturating concentration of this substrate (6.6 mM). A low ratio form of pyruvate kinase (ratio between 0.1 and 0.2) is obtained from livers perfused with 10^{-7} M glucagon or 0.1 mM adenosine 3':5'-monophosphate (cyclic AMP). A high ratio form of the enzyme is obtained from livers perfused with no hormone (ratio = 0.35 to 0.46). The regulation of pyruvate kinase by glucagon and cyclic AMP occurs within 2 min following the hormone addition to the liver. Insulin (22 milliunits/ml) counteracts the inhibition of pyruvate kinase caused by 5 x 10^{-6} M glucagon, but has only a slight influence on the enzyme properties in the absence of the hyperglycemic hormone.

The low ratio form of pyruvate kinase obtained from livers perfused with glucagon or cyclic AMP is unstable in liver extracts and will revert to a high ratio form within 10 min at 37\(^\circ\) or within a few hours at 0\(^\circ\). Pyruvate kinase is quantitatively precipitated from liver supernatants with 2.5 M ammonium sulfate. This precipitation stabilizes the enzyme and preserves the kinetically distinguishable forms.

The kinetic properties of the two forms of rat hepatic pyruvate kinase are examined using ammonium sulfate precipitates from the perfused rat liver. At pH 7.5 the high ratio form of the enzyme has \([S]_{0.5} = 1.6 \pm 0.2\) mM P-enolpyruvate \((n = 8)\). The low ratio form of enzyme from livers perfused with glucagon or cyclic AMP has \([S]_{0.5} = 2.5 \pm 0.4\) mM P-enolpyruvate \((n = 8)\). The modification of pyruvate kinase induced by glucagon does not alter the dependence of the enzyme activity on ADP \((K_A = 0.5\) mM ADP for both forms of the enzyme). Both forms are allosterically modulated by fructose 1,6-bisphosphate, L-alanine, and ATP.

The changes in the kinetic properties of hepatic pyruvate kinase which follow treating the perfused rat liver with glucagon or cyclic AMP are consistent with the changes observed in the enzyme properties upon phosphorylation \textit{in vitro} by a cyclic AMP-stimulated protein kinase (Ljungström, O., Hjelmquist, G. and Engström, L. (1974) Biochim. Biophys. Acta 358, 289-298). However, other factors also influence the enzyme activity in a similar manner and it remains to be demonstrated that the regulation of hepatic pyruvate kinase by glucagon and cyclic AMP \textit{in vivo} involves a phosphorylation.

The regulation of gluconeogenesis has been an area of intensive biochemical investigation for several years. Early studies of the stimulation of gluconeogenesis by glucagon in the perfused rat liver indicated that regulatory events occur at early reactions in the pathway, between pyruvate and P-enolpyruvate (1, 2). Recent studies, however, have shown this hyperglycemic hormone also inhibits hepatic glycolysis (3-5). It has been suggested that the hormonal regulation of hepatic carbohydrate metabolism involves a complex regulation of several gluconeogenic and glycolytic enzymes (3).

The pathways of glycolysis and gluconeogenesis share common enzymes; but enzymes unique to each process are found at several physiologically irreversible steps. The key enzymes which are candidates for regulation include: pyruvate carboxy-
lase, P-enolpyruvate carboxykinase, pyruvate kinase, phosphofructokinase, fructose-1,6-bisphosphatase, glucokinase, and glucose-6-phosphatase. Although these enzymes possess kinetic and allosteric properties which appear to be suited to their roles in hepatic carbohydrate metabolism, many events occurring with the hormonal regulation of this process can not be explained by these properties (3). The possibility exists that the hormonal regulation of hepatic carbohydrate metabolism involves a phosphorylation of several key gluconeogenic and glycolytic enzymes similar to the hormonal regulation of enzymes of glycogen metabolism (6).

Taunton and his co-workers (7–10) first reported in 1972 that glucagon rapidly increases the activity of hepatic fructose-1,6-bisphosphatase in the rat and decreases the activity of phosphofructokinase and pyruvate kinase. They also found insulin has actions opposite to glucagon. The modulation of the enzyme activities reported by these investigators is consistent with a concerted phosphorylation of several hepatic enzymes being regulated by glucagon (7–10); however, further characterization of this regulation and isolation of phosphorylated forms of the enzymes has not been reported.

Studies were undertaken in this laboratory concerning the hormonal regulation of various hepatic enzymes in an effort to extend the observations of Taunton et al. (7–10). We found injection of hormones into the anesthetized rat using procedures similar to those of Taunton et al. (7) does not provide a reproducible method for demonstrating the regulation of hepatic enzymes. However, we find a reproducible regulation of hepatic pyruvate kinase (ATP:pyruvate phosphotransferase, EC 2.7.1.40) by glucagon, cyclic AMP, and insulin, using the perfused rat liver. The present report concerns this regulation of hepatic pyruvate kinase and provides evidence for the existence of at least two kinetically distinct forms of the enzyme whose interconversion is regulated by glucagon and insulin. A preliminary report of this work has been presented (11).

MATERIALS AND METHODS

Male Wistar rats (Hilltop Laboratories weighing 250 to 350 g) were used. The rats were fed standard laboratory chow (Wayne) ad libitum or fasted 24 hours as indicated. The rats were anesthetized with pentobarbital (50 mg/kg, intraperitoneal) and each liver was surgically isolated and perfused with 100 ml of recirculating Krebs-Ringer bicarbonate buffer containing 3 g/100 ml of bovine serum albumin (Pentex, Fraction V) and 20 ml of washed bovine erythrocytes/100 ml of buffer (3, 12). Each liver was perfused for 60 min as an equilibration period before the experiment was initiated. For perfusions with livers from fasted rats, 10 mM l-lactate (Pfanstiehl) was added to the perfusate as a gluconeogenic precursor at 60 min. Other indicated additions were also made at this time and the perfusion was continued for an additional 30 min as the experimental period. Livers from fed rats received no gluconeogenic precursor and were given only those additions indicated under “Results.”

The rate of glucose synthesis by the perfused liver was determined by measuring glucose concentration in the perfusate at intervals during the perfusion. Perfusate samples for glucose analysis were deproteinized with perchloric acid (0.5 M final concentration) and glucose was determined by the glucose oxidase method (Boehringer Mannheim). Glucose production over the experimental period was linear unless otherwise noted.

For enzyme analysis, liver samples were homogenized with a Kontes-Dual homogenizer in ice-cold buffer at pH 7.5 containing 20 mM Tris-HCl, 120 mM KCl, 5 mM MgSO_4_2 and 0.1 mM Na_2EDTA (8). Approximately 20 ml of buffer was used to homogenize 1 g of liver in experiments measuring enzyme activity in total homogenates. For partial purification of pyruvate kinase the extraction volume was 4 ml of buffer/g of liver.

Pyruvate kinase assays were conducted with a thermostated Gilford recording spectrophotometer by coupling the enzyme activity to lactate dehydrogenase, using a procedure modified from that reported by Carminatti et al. (13). For studies using liver homogenates as the enzyme source, the assay medium at pH 7.5 contained 130 mM Tris-Cl, 68 mM KCl, 10 mM MgSO_4_2, 2.5 mM ADP, 0.16 mM NADH, 3.2 units/ml of lactate dehydrogenase (Boehringer Mannheim), and the indicated concentration of P-enolpyruvate (tricyclohexylammonium salt, Grand Island Biological Co.). The final assay volume was 0.65 ml and the assay temperature was 25°C unless otherwise noted. Pyruvate kinase activity in rat liver homogenates is not proportional to the amount of homogeneous protein added to the assay. Below 100 µg of homogenate protein/ml in the assay, little or no activity of the enzyme can be measured. From 180 to 900 µg/ml, the activity is linear with protein concentration. All assays for pyruvate kinase presented in this report were conducted with quantities of tissue extract which give enzyme activities proportional to protein concentration. For kinetic studies the assay medium at pH 7.5 was used unless otherwise specified. Varying concentrations of ADP and ATP (Boehringer Mannheim) were added to the assay medium as the MgCl₂ salts by mixing the diosyl salts with equimolar concentrations of MgSO_4.

The concentrations of P-enolpyruvate (14), ADP (15), fructose 1,6-bisphosphate (16), and ATP (17) in stock solutions were determined by measuring glucose concentration in the perfusate at intervals during the perfusion. The ATP content of the commercial ADP (Boehringer Mannheim) used in these studies was less than 2%. L-Alanine and 2-deoxyglucose were obtained from Sigma Chemical Co. Crystalline porcine glucagon and trypsin-treated, crystalline porcine insulin were a generous gift of Dr. Ronald Chance (Eli Lilly and Co., Indianapolis). These hormones were dissolved in 1 mM HCl before addition to the perfusion medium. In perfusions receiving no hormone, appropriate amounts of 1 mM HCl were added as a control treatment. Cyclic AMP (free acid, Sigma) was dissolved directly in the perfusion medium. All other reagents were of the highest grade commercially available. Protein was estimated by the method of Lowry et al. (18).

The data is expressed as the mean ± S.D. of multiple determinations where applicable. Statistical analysis was done by the Student’s t test. Kinetic studies presented as single experiments have been confirmed with at least two separate enzyme preparations.

RESULTS

Hormonal Regulation of Hepatic Pyruvate Kinase—Measurements of pyruvate kinase activity in homogenates prepared from the perfused rat liver were done using small tissue samples taken before and after treating the organ with glucagon. A rapid change in the enzyme activity resulting from the action of glucagon is observed if pyruvate kinase assays are conducted with a subsaturating concentration of P-enolpyruvate (1.3 mM, Table I). The activity of the enzyme determined at 1.3 mM P-enolpyruvate in homogenates from livers perfused with 10⁻³ M glucagon is approximately 50% the activity found in homogenates from the same liver prepared immediately prior to the hormone addition. The decrease in pyruvate kinase activity is observed within 2 min after adding glucagon to the perfusate and persists for more than 30 min. The regulation of pyruvate kinase by glucagon is observed with livers from fed and fasted rats and can be correlated with the hormonal stimulation of glycogenolysis and gluconeogenesis, respectively (Table I). Treating the perfused liver with 0.1 mM cyclic AMP causes a change in the activity of pyruvate kinase which is similar to the change induced by glucagon (Table I).

When pyruvate kinase activity is determined with 6.6 mM P-enolpyruvate the hormone influence is not detected (Table I). This high concentration of the substrate gives maximal activity of the enzyme in liver homogenates. The regulation of pyruvate kinase by glucagon, therefore, does not alter the total enzyme content in the liver, but rather appears to influence the dependence of the enzyme activity on P-enolpyruvate concentrations. This regulation of the substrate dependence is readily
Livers from rats fasted 24 hours and rats fed ad libitum were perfused 60 min with no additions as an equilibration period. At 60 min, livers from fasted rats were given 10 mM L-lactate as a gluconeogenic precursor and other additions as indicated in the table. Livers from fed rats received only those additions indicated in the table. Glucose production was linear over the next 30 min of perfusion and is expressed as micromoles/hour/100 g rat body weight. Liver samples were taken at 60 min, before any addition to the perfusate and at various times following the indicated additions for up to 30 min. The activity of pyruvate kinase was determined in homogenates using 1.3 and 6.6 mM P-enolpyruvate as described under “Methods and Materials” and is expressed as micromoles of pyruvate formed/min/mg of homogenate protein. The assay temperature was 37° for the enzyme obtained from livers of the fasted rats. This temperature was reduced to 25° for the experiments with livers from fed rats. The activity of pyruvate kinase found at various times during the perfusion, but with similar additions to the perfusate, have been combined. Glucose production is given as the mean ± S.D. with the number of perfusions shown in parentheses. Pyruvate kinase activity is presented as the mean ± S.D.; the number of liver samples examined is given in the last column of the table.

Pyruvate kinase activity and glucose production in perfused rat liver

<table>
<thead>
<tr>
<th>Additions</th>
<th>Glucose production</th>
<th>Pyruvate kinase activity at:</th>
<th>Ratio&lt;sup&gt;a&lt;/sup&gt;</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>µmol/hr/100 g</td>
<td>P-enolpyruvate</td>
<td>µmol/min/mg</td>
<td>1.3 mM P-enolpyruvate</td>
</tr>
<tr>
<td>Fasted&lt;sup&gt;b&lt;/sup&gt;</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control&lt;sup&gt;c&lt;/sup&gt;</td>
<td>184 ± 27 (6)</td>
<td>0.274 ± 0.084</td>
<td>0.118 ± 0.047</td>
<td>0.43</td>
</tr>
<tr>
<td>Glucagon, 1.4 × 10&lt;sup&gt;-4&lt;/sup&gt; M</td>
<td>206 ± 67 (6)&lt;sup&gt;d&lt;/sup&gt;</td>
<td>0.255 ± 0.085</td>
<td>0.070 ± 0.004&lt;sup&gt;e&lt;/sup&gt;</td>
<td>0.27</td>
</tr>
<tr>
<td>Fed&lt;sup&gt;b&lt;/sup&gt;</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control&lt;sup&gt;c&lt;/sup&gt;</td>
<td>39 ± 45 (7)</td>
<td>0.178 ± 0.031</td>
<td>0.062 ± 0.014</td>
<td>0.35</td>
</tr>
<tr>
<td>Glucagon, 1.4 × 10&lt;sup&gt;-4&lt;/sup&gt; M</td>
<td>671 ± 150 (9)&lt;sup&gt;d&lt;/sup&gt;</td>
<td>0.185 ± 0.044</td>
<td>0.030 ± 0.005&lt;sup&gt;e&lt;/sup&gt;</td>
<td>0.16</td>
</tr>
<tr>
<td>Cyclic AMP, 0.1 mM</td>
<td>616 ± 213 (8)&lt;sup&gt;d&lt;/sup&gt;</td>
<td>0.184 ± 0.025</td>
<td>0.030 ± 0.006&lt;sup&gt;e&lt;/sup&gt;</td>
<td>0.16</td>
</tr>
<tr>
<td>Insulin, 22 milliunits/ml</td>
<td>18 ± 57 (6)</td>
<td>0.188 ± 0.018</td>
<td>0.078 ± 0.008&lt;sup&gt;e&lt;/sup&gt;</td>
<td>0.40</td>
</tr>
</tbody>
</table>

<sup>a</sup>Activity at 1.3 mM P-enolpyruvate/activity at 6.6 mM P-enolpyruvate.

<sup>b</sup>Pyruvate kinase assayed at 37°.

<sup>c</sup>This group received either no additions or an appropriate control vehicle addition.

<sup>d</sup>p < 0.05 versus control.

<sup>e</sup>Pyruvate kinase assayed at 25°.

<sup>f</sup>p < 0.001 versus control.

*P<sub>enolpyruvate to that found at 6.6 mM P-enolpyruvate (Table I). This ratio may be determined directly without measuring the protein content of homogenates and has been useful for rapidly detecting the hormone influence during actual experiments. To facilitate further discussion, the enzyme obtained from livers perfused with glucagon or cyclic AMP will be referred to as the low ratio form of the enzyme. The enzyme form obtained from livers perfused with no hormone will be referred to as the high ratio form.

The low ratio form of pyruvate kinase is induced by glucagon concentrations in the perfusate as low as 5 x 10<sup>-6</sup> M (Fig. 1); below 10<sup>-10</sup> M, glucagon will not modulate the enzyme activity.

Insulin (22 milliunits/ml of final concentration) alone does not significantly influence the rate of glucose synthesis in perfused livers from fed rats (Table I). This hormone only slightly increases the activity of pyruvate kinase above the activity found in homogenates from control livers perfused with no hormone (approximately 20% at 1.3 mM P-enolpyruvate, Table I). More pronounced effects of insulin are observed in livers which have been treated with low concentrations of glucagon (5 x 10<sup>-11</sup> M, Fig. 1). Glucose synthesis in livers from fed rats receiving 5 x 10<sup>-10</sup> M glucagon was 325 ± 140 µmol/hour/100 g of rat body weight (n = 5).<sup>4</sup> The rate for livers receiving glucagon followed by 22 milliunits/ml insulin was significantly lower, being 103 ± 150 µmol/hour/100 g of body weight (n = 5, p < 0.05). This inhibition of glucose synthesis by livers perfused with glucagon followed by insulin.

<sup>4</sup>Glucone synthesis by livers perfused with 5 x 10<sup>-11</sup> M glucagon was not linear with time and was determined over the final 15 min of perfusion. This time period was also used to determine the rate of glucose synthesis by livers perfused with glucagon followed by insulin.

**Fig. 1.** Insulin reversal of the glucagon inhibition of hepatic pyruvate kinase. Livers from fed rats were perfused for 60 min as an equilibration period. At 60 min a liver sample was excised and glucagon was added to the perfusate to give a final concentration of 5 x 10<sup>-11</sup> M. Five minutes later another liver sample was excised and either 22 milliunits/ml of insulin (---) or a control vehicle (-----) was added to the perfusate. Additional liver samples were taken as shown in the figure. Pyruvate kinase was assayed at 1.3 and 6.6 mM P-enolpyruvate. The ratio of the activity of the enzyme at 1.3 mM P-enolpyruvate to that found at 6.6 mM P-enolpyruvate is shown. The data represent the mean ± S.D. of six perfusions: three livers receiving glucagon followed by insulin and three livers receiving glucagon followed by the control vehicle.
by insulin is accompanied by a reactivation of pyruvate kinase activity in homogenates when assays are conducted with 1.3 mM P-enolpyruvate (Fig. 1). Insulin does not overcome the influence of glucagon if the concentration of the hyperglycemic in the perfusate is raised above $10^{-19}$ M (data not shown).

**Pyruvate Kinase in Vivo**—The ratio of pyruvate kinase activity at 1.3 mM P-enolpyruvate to the activity at a saturating concentration of the substrate was determined in homogenates of livers taken from intact rats maintained on various diets. This ratio was $0.19 \pm 0.04$ ($n = 6$) for rats fed a standard laboratory chow and $0.16 \pm 0.03$ ($n = 4$) for rats fasted 48 hours. Feeding rats high carbohydrate or low carbohydrate diets also does not significantly alter the ratio from that found after 60 min. In the same liver the ratio promptly dropped to 0.19 after 0.1 mM cyclic AMP was added to the perfusate.

**Instability of Low Ratio Form of Hepatic Pyruvate Kinase**—The low ratio form of pyruvate kinase obtained from livers perfused with glucagon or cyclic AMP is quite unstable in liver extracts. This form of the enzyme (ratio between 0.1 and 0.2) will completely revert to the high ratio form (ratio between 0.35 and 0.45) within 4 hours at 0°C or within 10 min at 37°C, with no change in the maximal activity of the enzyme. The high ratio form of the enzyme is completely stable under these conditions. Because of this reversion, assays of pyruvate kinase were initiated as soon as possible after preparing homogenates, generally within 10 min. The assay temperature of 37°C used in early parts of the study was reduced to 25°C to minimize the reversion during the assay.

The hormonally modified forms of the enzyme are stabilized by precipitating the enzyme with 2.5 M ammonium sulfate (see the legend of Fig. 2 for details of the precipitation). This precipitation gives quantitative recovery of the pyruvate kinase activity and approximately a 2.5-fold purification. The two forms of hepatic pyruvate kinase are stable as precipitates in 2.5 M ammonium sulfate and may be kept for over 6 weeks with no detectable loss of activity.

**Substrate Dependence of Hepatic Pyruvate Kinase**—To further study the hormonal regulation of hepatic kinase, the kinetic properties of the two forms of the enzyme obtained from the perfused rat liver were investigated. These studies were conducted using ammonium sulfate precipitates of liver extracts and represent the information obtained with eight preparations of the high ratio form generated by glucagon or cyclic AMP.

Hepatic pyruvate kinase is homotropically activated by the substrate, P-enolpyruvate (Fig. 2). The concentration of P-enolpyruvate which gives 50% maximal activity ([$\text{PEP}]_{50}$) is dependent on experimental circumstances and several conditions have been examined in the present investigation. At pH 7.5 and 9.5 mM ADP, [$\text{PEP}]_{50}$ for the high ratio form of the enzyme from livers perfused with no hormone treatment is 1.6 ± 0.2 mM P-enolpyruvate ($n = 8$; Fig. 2). [$\text{PEP}]_{50}$ for the low ratio form of pyruvate kinase from livers perfused with glucagon or cyclic AMP is 2.5 ± 0.4 mM P-enolpyruvate ($n = 8$). At pH 7.1 in the presence of 10 mM ADP, similar to the conditions used by Taunton et al. (9), the [$\text{PEP}]_{50}$ is approximately 1.8 mM P-enolpyruvate and 4.2 mM P-enolpyruvate for the high and low ratio forms of the enzyme, respectively (see Fig. 2).

The dependence of pyruvate kinase activity on ADP was also examined. At saturating concentrations of P-enolpyruvate (7.5 mM), the enzyme has a hyperbolic dependence on varying ADP concentrations with $K_m$ being 0.47 ± 0.01 mM ADP ($n = 4$). No difference in the ADP dependence was observed between the two forms of the enzyme (not shown). We observed an inhibition of rat hepatic pyruvate kinase by ADP very similar to that reported for the pig liver enzyme by Kutzback et al.
observed that glucose + hexokinase cannot be used as an ATP "trap" because the enzyme becomes fully activated. In the latter case, it is not known if the activator is glucose-6-P or fructose 1,6-bisphosphate. Unpurified enzyme preparations used in these experiments, it is, without changing the control activity of the enzymes. With the hexokinase "trap" will abolish inhibition of the enzyme by 0.3 mM ATP. Experiments presented in Table II, it was found that the deoxyglucose-hepatic pyruvate kinase, but concentrations greater than 5 mM are produced during the reaction.

We observed that 2-deoxyglucose-6-P will allosterically activate the enzyme (for a review see Ref. 24). Both the low ratio and high ratio forms of pyruvate kinase are activated by fructose 1,6-bisphosphate (Fig. 3). We have not been able to detect any effect of deoxyglucose-6-P was observed below 0.5 mM. For the experiments presented in Table II, it was found that the deoxyglucose-hexokinase "trap" will abolish inhibition of the enzyme by 0.3 mM ATP without changing the control activity of the enzymes. With the unpurified enzyme preparations used in these experiments, it is observed that glucose + hexokinase cannot be used as an ATP "trap" because the enzyme becomes fully activated. In the latter case, it is not known if the activator is glucose-6-P or fructose 1,6-bisphosphate produced during the reaction.

### Table II

<table>
<thead>
<tr>
<th>Nucleotide</th>
<th>Additions</th>
<th>Pyruvate kinase activity at:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.5 mM ADP</td>
<td>2.7 mM ADP</td>
</tr>
<tr>
<td></td>
<td>units/mg</td>
<td>units/mg</td>
</tr>
<tr>
<td><strong>High ratio form</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MgADP</td>
<td>None</td>
<td>0.35</td>
</tr>
<tr>
<td></td>
<td>Hexokinase + 2-deoxyglucose</td>
<td>0.34</td>
</tr>
<tr>
<td></td>
<td>1 mM EGTA*</td>
<td>0.40</td>
</tr>
<tr>
<td>Na₂ADP</td>
<td>None</td>
<td>0.36</td>
</tr>
<tr>
<td></td>
<td>&quot;Dowex&quot; treated</td>
<td>0.35</td>
</tr>
<tr>
<td><strong>Low ratio form</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MgADP</td>
<td>None</td>
<td>0.22</td>
</tr>
<tr>
<td></td>
<td>Hexokinase + 2-deoxyglucose</td>
<td>0.24</td>
</tr>
<tr>
<td></td>
<td>1 mM EGTA*</td>
<td>0.26</td>
</tr>
<tr>
<td>Na₂ADP</td>
<td>None</td>
<td>0.22</td>
</tr>
<tr>
<td></td>
<td>&quot;Dowex&quot; treated</td>
<td>0.22</td>
</tr>
</tbody>
</table>

* Ethylene glycol bis(β-aminoethyl ether)-N,N'-tetraacetic acid.

This inhibition is primarily observed at suboptimal concentrations of P-enolpyruvate and is the result of high concentrations of ADP changing the P-enolpyruvate dependence of the enzyme (see Fig. 12 and Table 6 in Ref. 20). At 1.3 mM P-enolpyruvate, we observed optimal activity of the rat hepatic pyruvate kinase with approximately 0.5 mM ADP. Raising ADP from 0.5 to 2.7 mM decreases the activity by approximately 30% (Table II). We consistently observed that high concentrations of ADP inhibit the low ratio form of pyruvate kinase to a greater extent than the high ratio form of the enzyme (Table II); the difference, however, has been small and the significance of this observation is not presently known.

The inhibition by ADP is not due to ATP contamination nor to ATP production in the assay since introduction of an ATP "trap" does not relieve the inhibition (Table II). The inhibition is also observed when ADP is added as the disodium salt without compensating for Mg⁺⁺ complexed by the nucleotide. Other investigators have reported an inhibition of type L pyruvate kinase by ADP (13, 20–23), but not of the type M isozyme found in muscle (21–23). Carminatti et al. (13) suggest the inhibition is due to Cu⁺⁺ contamination of commercial ADP. We find the inhibition is not relieved by chelating agents nor by treating the ADP with a cationic exchange resin (Dowex 50, Table II). Further studies will be necessary to determine if the inhibition is a direct effect of the nucleotide on pyruvate kinase or is due to a contaminant which specifically alters the P-enolpyruvate dependence of the enzyme.

#### Allosteric Modifiers of Hepatic Pyruvate Kinase—Fructose 1,6-bisphosphate

Fructose 1,6-bisphosphate is an allosteric activator of hepatic pyruvate kinase and alanine and ATP are allosteric inhibitors of the enzyme (for a review see Ref. 24). Both the low ratio and high ratio forms of pyruvate kinase are activated by fructose 1,6-bisphosphate (Fig. 9). We have not been able to detect any difference in the P-enolpyruvate dependence of the two forms of the enzyme in the presence of optimal amounts of fructose 1,6-bisphosphate (15 μM, Fig. 3) which contrasts with the P-enolpyruvate dependence of the two forms in the absence of the activator (Fig. 2). The activation of pyruvate kinase by fructose 1,6-bisphosphate is reversible and the original kinetic forms of the enzyme are recovered following precipitation of the enzyme with ammonium sulfate to remove the hexose bisphosphate.

Alanine (Fig. 4A) and ATP (Fig. 4B) are allosteric inhibitors of both forms of hepatic pyruvate kinase. At fixed concentrations of P-enolpyruvate, the low ratio form of the enzyme is

![Fig. 3. P-enolpyruvate dependence of pyruvate kinase in the presence of 15 μM fructose 1,6-bisphosphate. Ammonium sulfate precipitates containing the high ratio form of pyruvate kinase (●, △) and the low ratio form of the enzyme (○, □) were prepared from a liver perfused with no hormone treatment and a liver perfused with 1.4 x 10⁻⁵ M glucagon, respectively, as described in the legend of Fig. 2. Assays were conducted at pH 7.5 with 2.5 mM ADP and the indicated concentrations of P-enolpyruvate (TEP). The P-enolpyruvate dependence was examined in the absence (△, □) and in the presence (●, ○) of 15 μM fructose 1,6-bisphosphate. Each point represents the average of duplicate determinations.](http://www.jbc.org/)

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inhibited more strongly by both alanine (Fig. 4A) and ATP (Fig. 4B) than is the high ratio form of the enzyme. A consideration of the heterotropic interactions between the substrate and the allosteric modifiers of the enzyme (25) and more detailed studies of the P-enolpyruvate dependence of the two enzyme forms in the presence of the inhibitors (not shown) suggest the quantitative differences in the response of the two enzyme forms to alanine or ATP is primarily due to the different $[\text{PEP}]_{50}$ of these forms.

**DISCUSSION**

One interpretation of the present finding is that the regulation of hepatic carbohydrate metabolism by glucagon and insulin includes a reversible interconversion of two kinetically distinct forms of hepatic pyruvate kinase. The existence of these distinct forms of the enzyme is indicated by the data presented in Table I and Fig. 2. Treating the perfused rat liver with glucagon or cyclic AMP rapidly produces a form of the enzyme whose activity at subsaturating concentrations of P-enolpyruvate (1.3 mM, Table I) is lower than the activity of the enzyme measured from livers perfused with no hormone treatment. The change in the enzyme activity caused by glucagon, however, is not apparent when the enzyme assays are conducted with a saturating concentration of the substrate (6.6 mM, Table I). More detailed studies of the kinetic properties of the enzyme in ammonium sulfate precipitates from these livers show the regulation involves a modification of the enzyme dependence on P-enolpyruvate (Fig. 2). For the present discussion, the two forms of the enzyme may be distinguished by the ratio of activity at 1.3 mM P-enolpyruvate to the activity at 6.6 mM P-enolpyruvate (Table I). The low ratio form of the enzyme obtained from livers perfused with glucagon or cyclic AMP has $[\text{PEP}]_{50}$ of 2.5 mM P-enolpyruvate at pH 7.5 in the presence of 2.5 mM ADP. A high ratio form of the enzyme having $[\text{PEP}]_{50}$ of 1.6 mM P-enolpyruvate is obtained from livers perfused with no hormone or livers perfused with insulin. The hormonal regulation does not alter the dependence of hepatic pyruvate kinase on ADP (see text). Studies of the response of the forms of pyruvate kinase to the allosteric effectors fructose 1,6-bisphosphate (Fig. 3), L-alanine (Fig. 4A), and ATP (Fig. 4B) indicate that the hormonal regulation does not qualitatively change the allosteric properties of the enzyme (i.e. by eliminating a particular allosteric response). Quantitative differences in the allosteric response of the two enzyme forms to alanine and ATP (Fig. 4) appear mainly due to the different $[\text{PEP}]_{50}$ of the two enzyme forms. It should be noted that the hormonal regulation of the enzyme will not be observed when assays are carried out in the presence of the allosteric activator, fructose 1,6-bisphosphate (Fig. 3).

The regulation of pyruvate kinase by glucagon and cyclic AMP correlates with the stimulation of glucose synthesis in the perfused rat liver (see Table I) and the inhibition of hepatic glycolysis by these agents (3-5). Park and Exton (2) have discussed the influence of insulin on glucose synthesis in the perfused rat liver in terms of the hypoglycemic hormone lowering intracellular concentrations of cyclic AMP. They suggest the perfusion process lowers cyclic AMP concentrations to an extent that insulin has no impact on glucose synthesis unless cyclic AMP concentrations are first elevated by glucagon. If cyclic AMP concentrations are excessively elevated by high concentrations of glucagon, insulin will then not overcome the influence of the hyperglycemic hormone. Since the active pool (unsequestered pool, Ref. 2) of cyclic AMP may be much less than the total amount of cyclic AMP determined in liver extracts, this proposed mechanism has been experimentally difficult to confirm by measuring total tissue levels of the cyclic nucleotide (see Ref. 2 for a discussion). The present observations concerning the influence of perfusing the liver (see text) and the influence of insulin on

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Fig. 4. Inhibition of pyruvate kinase by L-alanine and ATP. A high ratio form (O, A) and a low ratio form (O, △) of pyruvate kinase were prepared as described in the legend of Fig. 2. Assays were conducted at pH 7.5 with 2.5 mM ADP and either 4.8 mM P-enolpyruvate (PEP) (O, △) or 6.9 mM P-enolpyruvate (△, △). The inhibition by various concentrations of L-alanine is shown in A and by various concentrations of ATP is presented in B. Each point represents the average of duplicate determinations.

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*The value found for this ratio is influenced by assay conditions and may change with purification of the enzyme. The designation of high and low ratio forms of the enzyme applies only to the enzyme determined in unpurified liver homogenates at pH 7.5 and 25° with 9.5 mM ADP present. Characterization of the two forms in more purified preparations must rely on more detailed kinetic studies under defined conditions.*
pyruvate kinase activity in the perfused rat liver (Table I and Fig. 1) are consistent with the proposal of Park and Exton (2). Perfusing the liver results in the formation of the high ratio form of pyruvate kinase. Adding insulin to livers previously perfused with low concentration of glucagon will generate the high ratio form of the enzyme (Fig. 1); although the hypoglycemic hormone has only a small influence on the enzyme activity in livers not pretreated with glucagon (Table I). Above 10−16 M glucagon, insulin will not overcome the influence of the hyperglycemic hormone (see text). Coupled with the fact that cyclic AMP also generates the low ratio form of the enzyme, the above observations are consistent with a hypothesis that the hormonal regulation of hepatic pyruvate kinase is mediated by the intracellular “messenger,” cyclic AMP.

Ljungström and his co-workers (26, 27) recently reported that purified hepatic pyruvate kinase may be phosphorylated by a cyclic AMP-stimulated protein kinase in vitro. The influence of phosphorylation on the kinetic properties of the enzyme (26) are similar to the changes in the kinetic properties of the enzyme seen after treating the perfused liver with glucagon or cyclic AMP in the present investigation. It is tempting to extrapolate the in vitro phosphorylation of pyruvate kinase to the in vivo hormonal regulation of the enzyme. Such extrapolation, however, must be made with caution. Bylund and Krebs (28) have pointed out the protein kinases show low specificity for protein substrates and, in fact, may be more reactive with denaturated forms of certain proteins. Also the studies reported by Ljungström et al. (26) concerning the phosphorylation of pyruvate kinase indicate that treating pyruvate kinase with the protein kinase in the absence of ATP (presumably with no phosphorylation occurring) also alters the kinetic properties of pyruvate kinase (see Fig. 4 in Ref. 26). This discrepancy in their observations must be resolved before final conclusions can be reached concerning the effect of phosphorylation on the kinetic properties of the enzyme. Like many allosteric enzymes, type I pyruvate kinase is susceptible to modification by many experimental manipulations which change the kinetic properties of the enzyme. It is well established that the enzyme is sensitive to oxidizing agents (29, 30) and heavy metal ions (31, 31) which alter the dependence of the enzyme on P-enolpyruvate without influencing the maximal catalytic activity of the enzyme. The physiological significance of these in vitro effects is not known; however, it has been suggested that a regulation of the oxidation state of the enzyme may play an important role in regulating glycolysis in erythrocytes (30) and in the liver (30). We observe that chelating agents (Table II) do not abolish the difference between the kinetic properties of the low and high ratio forms of pyruvate kinase and we do not feel the regulation is due to changes in the ion content of the liver accompanying hormone treatment.

Because many factors may influence the kinetic properties of hepatic pyruvate kinase, confirmation of the proposal that the hormonal regulation of the enzyme involves a phosphorylation-dephosphorylation mechanism will depend on further purification and characterization of the two enzyme forms observed in the present investigation. The instability of the low ratio form, however, methods for stabilizing and isolating the two forms of the enzyme are currently being investigated in this laboratory. The present study indicates the perfused rat liver provides a good model for studying the hormonal regulation of pyruvate kinase and provides a means of correlating the in vivo regulation of the enzyme reported by Ljungström et al. (26, 27).

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