Effect of Insulin on Liver Pyruvate Kinase in Vivo and in Vitro*

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The relative rate of pyruvate kinase synthesis in alloxan-diabetic rats was ½ of that observed in controls and increased 5- to 4-fold upon treatment with insulin for 3 days. Similar changes in the catalytic activity were observed: 120 ± 40, 650 ± 100, and 230 ± 79 units of pyruvate kinase/liver in diabetic rats, diabetic rats maintained on insulin for 3 days, and controls, respectively. Thus, changes in the rate of synthesis of liver pyruvate kinase may be a significant mechanism which results in an altered level of liver pyruvate kinase activity in vivo.

The effect of insulin on liver pyruvate kinase levels was investigated in vitro by use of primary cultures of rat liver parenchymal cells. Hepatocytes freshly isolated from livers of control rats contained 14.1 ± 2.0 (n = 9) units of pyruvate kinase activity/mg of DNA. When maintained in a medium containing insulin (10^{-8} M), the pyruvate kinase activity of hepatocytes remained constant throughout the 7- to 14-day culture periods. In contrast, in cells maintained in the absence of insulin, the pyruvate kinase activity decreased progressively with time with an apparent first order decay half-life of 2 days. Hepatocytes from diabetic rats initially have a low pyruvate kinase activity, 3.2 units/mg of DNA. When these cells were maintained in medium containing 10^{-6} M insulin, the pyruvate kinase activity remained low for the initial 3 days, then rapidly increased 7-fold between days 3 and 7 and finally decreased back to normal levels by day 11. The relative rate of liver pyruvate kinase synthesis remained constant for 2 days, increased 3-fold on day 3, and remained elevated throughout the remainder of the 7-day culture period. Likewise, administration of insulin in vivo had increased the relative rate of liver pyruvate kinase synthesis; however, effects could be seen within 1 day. The more rapid response of liver pyruvate kinase to insulin in vivo may be the result of an altered systemic endocrine status in addition to a direct effect of insulin on the pyruvate kinase activity in liver cells. The 2- or 3-day lag period observed with hepatocytes from diabetic rats or control hepatocytes cultured 24 h in the absence of insulin may be due to the time required for the transcription, translation, and post-translational control of pyruvate kinase synthesis.

At least four isozymes of pyruvate kinase (ATP:pyruvate 2-O-phosphotransferase EC 2.7.1.40) are known and have been characterized by their kinetic, electrophoretic, immunologic, and chemical properties (for review, see Ref. 1). The L-type pyruvate kinase is the major isozyme in liver. It fluctuates in response to dietary and hormonal manipulations in vivo and in vitro and is believed to play a role in the regulation of carbohydrate metabolism. For instance, the administration of glucagon or epinephrine results in the phosphorylation of the L-type isozyme, decreasing its catalytic activity and increasing the $K_M$ for phosphoenolpyruvate (for review, see Ref. 2). However, the phosphorylation does not alter the $V_{max}$ of pyruvate kinase if the activity is assayed either with saturating concentrations of phosphoenolpyruvate or in the presence of the allosteric activator fructose 1,6-bisphosphate. The hormone-induced phosphorylation of liver pyruvate kinase occurs within minutes and does not result in a lower amount of enzyme protein.

The effect of insulin on liver pyruvate kinase has been investigated in several studies. The total liver pyruvate kinase activity decreased 40% when rats were rendered diabetic and increased back to normal values within 1 or 2 days of insulin therapy (3). Simultaneous administration of ethionine or actinomycin D effectively blocked the increase in pyruvate kinase activity and led to the suggestion that insulin treatment may induce the de novo synthesis of pyruvate kinase from newly synthesized mRNA (3). In rats pretreated with cortisol or a high protein diet to reduce the pyruvate kinase present, insulin administration resulted in increased enzyme activity (4). In 1969, Siller et al. (5) noted that when diets rich in fructose, glycerol, or both were fed to normal or diabetic rats for several days, the liver pyruvate kinase activity was high. Thus, they suggested that insulin administration resulted in a sequential induction of glucokinase and a secondary induction of liver pyruvate kinase by glycolytic intermediates (5). Rapid changes have also been reported; for instance, within minutes of administration, insulin reversed the glucagon-induced increase in the $K_M$ of pyruvate kinase for phosphoenolpyruvate in perfused rat liver but had little effect in controls without the glucagon pretreatment (6).

In the current investigation, the effect of insulin on the relative rate of liver pyruvate kinase synthesis was measured in vivo following a single intraperitoneal injection of [3H]leucine into diabetic rats, diabetic rats maintained on insulin, and control rats. Subsequently, hepatocytes under well defined conditions of culture were used to investigate the effect of insulin on liver pyruvate kinase and its relative rate of synthesis in vitro.

**EXPERIMENTAL PROCEDURES**

Male Sprague-Dawley rats (150 to 250 g, Holtzman Co., Madison, WI) given food and water ad libitum were used throughout these studies unless otherwise noted. Diabetes was induced by subcutaneous injection of a freshly prepared solution of alloxan, 20 mg of alloxan in 0.125 M citrate buffer, pH 4/100 g of body weight (7), into rats that had been fasted for 2 days. The animals having a glucosuria greater than 250 mg/dl 4 days after the alloxan injection were consid-
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To assay pyruvate kinase in the cultured hepatocytes, the medium was removed and the attached cells were washed three times with ice-cold Krebs-Ringer phosphate buffer, pH 7.4. The cells were then detached from the collagen gels by incubation for 20 min at 37°C in 2 ml of Krebs-Ringer phosphate buffer, pH 7.4, which contained 1 mg of collagenase per ml and 100 IU of plasminogen/ml. The detached cells were transferred to a centrifuge tube and collected by centrifugation for 5 min at 50 x g. The cells were then washed three times with Krebs-Ringer phosphate buffer, pH 7.4, and collected by centrifugation for 1 min at 100,000 x g. The final cell pellet was suspended in 1 ml of 0.5 ml of 30% hydrogen peroxide at 80°C for 4 h. The vials were cooled, 10 ml of Aquasol II were added, and radioactivity was determined by scintillation counting.

Separation of the pyruvate kinase isozymes was performed by thin layer polyacrylamide gel electrophoresis (19) and immunoprecipitation. Pyruvate kinase activity on the gel slab was detected by the method of Sosud and Rutter (20).

Relative Rate of Liver Pyruvate Kinase Synthesis in Vivo—Hepatocytes were isolated from diabetic rats and incubated as previously described. At 1-day intervals following the attachment of the cells to the collagen layers, the medium was removed, the cells were washed one time with medium prepared with methionine-free Leibovitz L-15, and the cells were incubated in 2 ml of fresh medium prepared with methionine-free Leibovitz L-15, 5 to 12.5 μCi of 18Cleucine (1024.4 Ci/mmol), 10^-6 M insulin, and other supplements previously noted. Leibovitz L-15 medium normally contains 150 mg of DL-methionine/liter (=1 ml ml-methionine). The methionine concentration in the incubations containing methionine-free medium and labeled methionine ranged from 2.4 to 6.1 mM. Four hours after the addition of the labeled methionine, the cells were harvested, washed, and lysed by sonication. The DNA content of the sonicated solution was measured (10). The incorporation of radioactivity into total trichloroacetic acid-precipitable protein and immunoprecipitated liver pyruvate kinase were measured as previously described.

RESULTS

Incorporation of [3H]leucine into Rat Liver Pyruvate Kinase in Vivo—A single pulse of [3H]leucine was administered by intraperitoneal injection into each diabetic rat, diabetic rat maintained on insulin, and control rat. Four hours after injection, their livers were rapidly removed and homogenized. The homogenates were assayed for pyruvate kinase activity and for the incorporation of radiolabel into total liver pyruvate kinase and total protein. As previously reported (3), the total liver pyruvate kinase activity in diabetic rats was approximately half that observed in controls and the total pyruvate kinase activity per liver increased 1.8-fold, 4.6-fold, and 5.4-fold during 1, 2, or 3 days of insulin administration to diabetic rats, respectively (Table I). The relative rate of synthesis of liver pyruvate kinase in the diabetic rats was 50% that observed in the controls and increased 3.5-fold upon treatment with insulin for 3 days. Thus, alteration of the rate of synthesis of liver pyruvate kinase could be a significant factor contributing to the amount of enzyme activity observed in normal, diabetic, and insulin-treated diabetic rats. To further investigate a possible direct role of insulin in the regulation of liver pyruvate kinase, primary cultures of rat hepatocytes were utilized.

Effect of Insulin on Liver Pyruvate Kinase in Primary Cultures of Rat Hepatocytes—A mean value of 14.1 ± 2.0 units of pyruvate kinase/mg of DNA was obtained for control cultures.

To assay pyruvate kinase in the cultured hepatocytes, the medium was removed and the attached cells were washed three times with ice-cold Krebs-Ringer phosphate buffer, pH 7.4. The cells were then detached from the collagen gels by incubation for 20 min at 37°C in 2 ml of Krebs-Ringer phosphate buffer, pH 7.4, which contained 1 mg of collagenase per ml and 100 IU of plasminogen/ml. The detached cells were transferred to a centrifuge tube and collected by centrifugation for 5 min at 50 x g. The cells were then washed three times with Krebs-Ringer phosphate buffer, pH 7.4, and collected by centrifugation for 1 min at 100,000 x g. The final cell pellet was suspended in 1 ml of 0.5 ml of 30% hydrogen peroxide at 80°C for 4 h. The vials were cooled, 10 ml of Aquasol II were added, and radioactivity was determined by scintillation counting.

Separation of the pyruvate kinase isozymes was performed by thin layer polyacrylamide gel electrophoresis (19) and immunoprecipitation. Pyruvate kinase activity on the gel slab was detected by the method of Sosud and Rutter (20).
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Table I
Relative rate of synthesis of pyruvate kinase in livers of alloxan-diabetic rats with and without insulin administration

Male rats in the condition indicated had food and water ad libitum. Diabetic animals (+insulin) were killed 4 or 7 days after alloxan administration. Diabetic rats being maintained on insulin were killed following 1, 2, or 3 days of insulin administration. All values are mean ± S.D. from n different animals.

<table>
<thead>
<tr>
<th>Condition</th>
<th>Liver pyruvate kinase activitya</th>
<th>Incorporation of radioactivityb (dpm in liver pyruvate kinase/dpm in soluble protein)</th>
<th>Relative rate of synthesis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>230 ± 79 (13)</td>
<td>1.54 ± 0.91 × 10⁻³ (n = 6)</td>
<td>1.0</td>
</tr>
<tr>
<td>Diabetic</td>
<td></td>
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<tr>
<td>-Insulin</td>
<td></td>
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<td></td>
</tr>
<tr>
<td>4 days</td>
<td>120 ± 40 (7)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7 days</td>
<td>135 (4)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>+Insulin</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 day</td>
<td>220 ± 50 (6)</td>
<td>5.66 ± 1.8 × 10⁻⁴ (n = 4)</td>
<td>0.37</td>
</tr>
<tr>
<td>2 days</td>
<td>550 ± 240 (8)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3 days</td>
<td>650 ± 110 (8)</td>
<td>1.96 ± 0.36 × 10⁻³ (n = 3)</td>
<td>1.3</td>
</tr>
</tbody>
</table>

a Total units of liver pyruvate kinase activity/liver.

b Rats in the condition indicated received a single injection of 1 mCi of L-[4,5-³H]leucine. Four hours after the injection, the rats were decapitated, the livers were rapidly removed and homogenized, and radioactivity incorporated into liver pyruvate kinase and total soluble protein were determined (disintegrations/min/g of liver) from n rats.

A rapid decrease in pyruvate kinase activity was observed when parenchymal cells were isolated from normal rats and were maintained in primary cultures in the absence of insulin (Fig. 1A). The decrease in enzyme activity follows an apparent first-order decay with a half-life of 2 days. In contrast, if the culture medium contained insulin, the liver pyruvate kinase activity was constant throughout the culture period. However, if cells isolated from a normal rat were cultured for 1 day in the absence of insulin prior to addition of the hormone to the medium, the pyruvate kinase activity continued to decrease through day 5, but subsequently returned to control values by day 8 of culture. When insulin was included in the culture medium, more cells apparently remained attached to the collagen (see Fig. 1B). However, in both the presence or absence of insulin, the amount of DNA per dish decreased similarly with time as some cells detached from the gels.

Fig. 2. Effect of insulin concentration on liver pyruvate kinase activity in primary cultures of hepatocytes maintained on collagen gels. Hepatocytes were isolated from a normal rat and maintained from day 0 in Leibovitz L-15 medium containing various concentrations of insulin noted and supplemented as described under "Experimental Procedures." The cells were maintained in a humidified incubator at 37 °C with medium changes every 24 h. The hepatocytes were harvested after 4 days and the pyruvate kinase activity was measured. Each point represents the mean value ± S.D. from three plates. EU, enzyme units.

A diabetic rat was investigated with the results illustrated in Fig. 3. The hepatocytes initially contained 3.2 units of pyruvate kinase activity/mg of DNA. Inclusion of 10⁻⁶ M or higher concentrations of insulin in the medium resulted in complete maintenance of the pyruvate kinase level during the 4 days of culture. Half-maximal effect was obtained with approximately 10⁻⁹ M insulin.
increased to a maximal response of 7-fold by day 7 and decreased to control values by day 11. The dramatic increase in the total liver pyruvate kinase seen in Fig. 3 could result from an increased level of enzyme synthesis. Thus, the relative rate of liver pyruvate kinase synthesis was investigated in vitro.

Incorporation of [3S]Methionine into Liver Pyruvate Kinase in Vitro—The time course of 3S incorporation into liver pyruvate kinase was investigated using hepatocytes isolated from a normal rat and attached to collagen layers as previously described. After the 4-h attachment period, the medium was removed and replaced with 2.0 ml of medium which contained methionine-free Leibovitz L-15 and [3S]methionine. Incubations were carried out at 37°C. Cells were harvested at 1-h intervals, washed, and lysed prior to determination of radiolabel incorporated into total protein and liver pyruvate kinase. The incorporation of radiolabel into both liver pyruvate kinase and total protein was proportional with time of incubation for at least 5 h (Fig. 4).

The effect of insulin on the relative rate of liver pyruvate kinase synthesis in vitro was investigated using hepatocytes which were isolated from a diabetic rat, attached to collagen layers, and maintained in culture medium containing 10 mM insulin. At 1-day intervals, the culture medium was removed and replaced with medium containing methionine-free Leibovitz L-15 and [35S]methionine. Incubations were continued for 4 h and then the cells were harvested, washed, and sonicated. The incorporation of radiolabel into total protein and liver pyruvate kinase was determined (Fig. 5A).}

**Fig. 3.** Effect of insulin on liver pyruvate kinase activity in hepatocytes isolated from a diabetic rat. Hepatocytes were isolated from a diabetic rat and were maintained in Leibovitz L-15 medium containing 10^{-6} M insulin and supplemented as described under “Experimental Procedures.” Cells were harvested at the time intervals noted and the liver pyruvate kinase (LPK) activity was determined. The data represent the mean ± S.D. from three plates. EU, enzyme units.

**Fig. 4.** Time course of incorporation of [35S]methionine into liver pyruvate kinase in vitro. Hepatocytes were isolated from a normal rat and attached to collagen layers. After the 4-h attachment period, the medium was removed, and the cells were washed one time with methionine-free medium and then incubated in 2.0 ml of medium containing methionine-free Leibovitz L-15, 5 μCi of [35S]methionine (1024 Ci/mmol), 10^{-6} M insulin and supplemented as described under “Experimental Procedures.” Cells were harvested at hourly intervals and the radiolabel incorporated into total protein and liver pyruvate kinase was determined. The incorporation of radiolado was essentially proportional with time of incubation up to the 5-h period investigated. The data represent the mean ± S.D. from three plates.

**Fig. 5.** Effect of insulin on the relative rate of liver pyruvate kinase synthesis in hepatocytes isolated from a diabetic rat. Hepatocytes were isolated from a diabetic rat and maintained in Leibovitz L-15 medium containing 10^{-6} M insulin and supplemented as described under “Experimental Procedures.” The cells were attached to collagen layers and at the time intervals noted, the medium was removed, and cells were washed once and then incubated in 2.0 ml of methionine-free Leibovitz L-15 medium containing 12.5 μCi of [35S]methionine (1024 Ci/mmol) and 10^{-6} M insulin and supplemented as described under “Experimental Procedures.” The incubation was continued for 4 h and then the cells were harvested, washed, and lysed. The radioactivity in total protein and liver pyruvate kinase was determined (A). The data are the mean of duplicate dishes of cells at each time point and each was within 5% of the mean value. The relative rate of liver pyruvate kinase synthesis was calculated on each day from the ratio of counts/min incorporated into liver pyruvate kinase/counts/min incorporated into total protein (see B).
to the 3-day lag period prior to an increase in catalytic activity (Fig. 3). As one might expect, the relative rate of liver pyruvate kinase synthesis increased prior to the increase in total enzyme activity.

It has been reported that the quantitative relationships between pyruvate kinase isozymes are altered during hepatocyte culture. Therefore, the amount of L-type isozyme of pyruvate kinase present throughout the culture period was quantitated by two methods, quantitative immunoprecipitation and electrophoresis. Hepatocytes were isolated from normal rats and maintained in a culture medium containing 10⁻⁶ M insulin and the percentage of pyruvate kinase activity that was immunoprecipitated by excess anti-liver pyruvate kinase immunoglobulin was measured. The immunoglobulin used would quantitatively immunoprecipitate purified rat liver pyruvate kinase (L-type). In contrast, 45% of the pyruvate kinase activity in rat kidney homogenate and no enzyme activity of rat femoral muscle homogenate was immunoprecipitated by the immunoglobulin. In freshly isolated parenchymal cells, 91% of the total pyruvate kinase activity was immunoprecipitated by the anti-liver pyruvate kinase immunoglobulin (Table II). Furthermore, the percentage of pyruvate kinase that was immunoprecipitated by anti-liver pyruvate kinase goat immunoglobulin decreased only slightly throughout a 12-day culture period. In addition, thin layer polyacrylamide gel electrophoresis was used to separate the pyruvate kinase isozymes which were detected by catalytic activity. Between 80 and 90% of the pyruvate kinase catalytic activity of freshly isolated parenchymal cells had an electrophoretic mobility similar to the purified rat liver (L-type) isozyme. Furthermore, the L-type isozyme comprised 80 to 90% of the pyruvate kinase activity throughout the 12-day culture period. Thus, it was concluded that the L-type isozyme of pyruvate kinase was the predominant form in these cell cultures and no radical changes in isozyme type were seen during the 12-day culture period.

Sillero et al. (5) have suggested that insulin administration to diabetic animals results in a normal induction of glucokinase and a secondary metabolite induction of pyruvate kinase. To examine this point, hepatocytes from normal animals were isolated and maintained in culture medium which contained various additional carbohydrates in the presence or absence of insulin and dexamethasone. After 4 days in culture, the cells were harvested and assayed for pyruvate kinase activity (Table III). In almost every instance, supplementation of the medium with fructose or glucose resulted in higher pyruvate kinase activity. The presence or absence of dexamethasone resulted in only slight differences in pyruvate kinase activity. However, approximately twice as much pyruvate kinase was observed in cells that had been cultured in medium containing insulin as compared to cells cultured in the same medium without insulin.

### DISCUSSION

Insulin is a potent hormone that influences a variety of metabolic functions in most tissues (21). In liver in vivo, insulin has an anabolic effect and influences the level of several specific proteins. Insulin administration to diabetic rats over several days decreases the level of enzymes involved in gluconeogenesis (pyruvate carboxylase, phosphoenolpyruvate carboxykinase, fructose-1,6-bisphosphatase, and glucose-6-phosphatase) and increases the level of enzymes involved in glycolysis (glucokinase, phosphofructokinase, and pyruvate kinase). Protein synthesis or degradation is ultimately required for these effects but there is a question of whether insulin acts directly as an inducer or repressor of synthesis of these enzymes.

The mechanism of insulin effects on liver metabolism has remained largely undefined because effects in vivo could not be easily reproduced in vitro. Insulin stimulates liver cell growth, glycogen deposition, and RNA and protein synthesis in diabetic rats as summarized by Steiner (22). However, the time course of the insulin-mediated effects were complex. 1) Effects of insulin on liver require hours or days to develop; 2) many effects appear to require specific RNA or protein synthesis; 3) effects in the liver are dependent upon the prior hormonal and nutritional status of the animal. For instance, increased levels of tyrosine aminotransferase messenger RNA were detected in vivo within 1 h after insulin administration to adrenalectomized rats (23). Glucokinase activity was induced upon insulin administration to diabetic animals and occurred over a 12- to 48-h time period (24). Maximal levels of liver RNA and RNA polymerase activity required 1 or 2 days to occur and the amounts of DNA and DNA polymerase activity were maximal 2 to 3 days after insulin administration.

### Table II

| Percentage of pyruvate kinase activity in hepatocytes immunoprecipitated by anti-liver pyruvate kinase goat immunoglobulin |  
| --- | --- |
| Hepatocytes were isolated from a normal rat and maintained in culture containing 10⁻⁶ M insulin. On the day indicated, the cells were harvested, sonicated, and centrifuged for 30 min at 100,000 X g. The supernatant fraction was assayed for pyruvate kinase activity and then 0.5 ml was incubated with an equivalent amount of anti-liver pyruvate kinase immunoglobulin for 24 h at 4 °C. The immunoprecipitate was removed from solution by centrifugation at 10,000 x g for 15 min and the supernatant fraction was assayed for pyruvate kinase activity. The mean ± S.D. of the percentage of pyruvate kinase activity immunoprecipitated from three separate experiments is presented. No enzyme activity was immunoprecipitated when pre-immune immunoglobulin was used as a control. |  
| Days in culture | Pyruvate kinase immunoprecipitated %  
| --- | ---  
| 0 | 91 ± 0.2  
| 2 | 90 ± 0.8  
| 4 | 89 ± 0.1  
| 6 | 85 ± 4.3  
| 9 | 86 ± 1.7  
| 12 | 82 ± 3.3  

### Table III

| Effect of various substrates on the liver pyruvate kinase activity in hepatocytes maintained ±insulin and ±dexamethasone  
| --- | ---  
<p>| Hepatocytes were isolated, attached to collagen gels as described under “Experimental Procedures,” and maintained in Leibovitz L-15 medium (Leibovitz L-15 medium contains 90 mg of galactose/dl) containing 100 IU of penicillin/ml, 100 μg of streptomycin/ml, 25 μg of fungizone/ml, 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffer, pH 7.4, 10% fetal bovine serum, ±10⁻⁶ M insulin, ±10⁻⁶ M dexamethasone. The medium also contained various carbohydrates (150 mg/dl) as noted. The cells were maintained at 37 °C in a humidified incubator with medium changes every 24 h. The cells were harvested after 4 days and the liver pyruvate kinase activity (units/mg of DNA) was determined. The values given are the mean ± S.D. from three plates. |</p>
<table>
<thead>
<tr>
<th>Additions</th>
<th>Pyruvate kinase activity</th>
</tr>
</thead>
</table>
| Insulin | No insulin  
| (+)Dexa-methasone | (-)Dexa-methasone | (+)Dexa-methasone | (-)Dexa-methasone |  
| No additions | 7.7 ± 0.2 | 8.1 ± 0.4 | 4.1 ± 0.9 | 4.8 ± 0.1 |  
| Glucose | 11.0 ± 0.4 | 11.8 ± 0.6 | 6.2 ± 1.6 | 4.7 ± 0.1 |  
| Fructose | 13.2 ± 0.7 | 11.8 ± 0.8 | 7.2 ± 0.9 | 6.4 ± 0.4 |  
| Glycoler | 10.6 ± 0.3 | 8.1 ± 0.1 | 6.2 ± 0.8 | 5.8 ± 0.4 |
to diabetic rats (22). In recent in vitro studies, glycogen synthesis, glycogen synthase activity, and RNA synthesis were reported to be elevated less than 1 h after insulin addition to hepatocytes isolated from diabetic rats (23). Hepatocytes bind and degrade insulin ($K_d = 3.5 \times 10^{-8}$ M) which led Steiner et al. (25) to propose that the fragments of the hormone might act directly as second messengers or by reinforcing the action of a receptor-generated second messenger.

Insulin could theoretically induce the rapid regulation of enzyme activity by alteration of metabolite levels. Park et al. (26) have hypothesized that many (but not all) of the hepatic actions of insulin can be accounted for by alteration in the intracellular level of cyclic AMP. Glucagon and catecholamines result in elevation of hepatic cyclic AMP levels and inhibition of pyruvate kinase activity. Insulin could counteract these effects by lowering cyclic AMP levels. Insulin has been reported to rapidly (within minutes) modulate the substrate dependence of liver pyruvate kinase in perfused rat livers which were pretreated with low doses of glucagon (6). It is tempting to consider that the mechanism of this rapid increase in pyruvate kinase activity in response to insulin would occur through dephosphorylation of the enzyme, since phosphorylation of liver pyruvate kinase has been reported to occur in response to glucagon or epinephrine. The increase in rat liver pyruvate kinase activity observed within 10 min after administration of insulin via portal vein (27) and the alteration in the ratio of pyruvate kinase activity within 15 to 30 min after adding insulin to a rat liver perfusion (6) would be consistent with alteration of the phosphorylation level of liver pyruvate kinase because the assays were performed under conditions which could detect changes in the catalytic activity resulting from a phosphorylation-dephosphorylation process. In contrast, the assays of pyruvate kinase performed in this current study were designed to measure the total enzymatic activity present. Thus, changes in catalytic activity observed were not a result of a phosphorylation-dephosphorylation mechanism.

Dexamethasone supplementation was used throughout these investigations. Dexamethasone supplementation has been reported to increase longevity and maintain the polygonal epithelial morphology in cultures of hepatocytes (28). Triamcinolone and dexamethasone has been reported to decrease rabbit liver pyruvate kinase activity (29, 30). However, in the experiments reported here (Table III), little difference in the pyruvate kinase activity was observed in the presence or absence of dexamethasone.

At least four isozymes of pyruvate kinase have been reported (19, 20, 31) and purified (32). The major isozyme of pyruvate kinase in liver is the L-type with smaller amounts of the M-type isozyme also being present. Parenchymal cells have been reported to contain only L-type isozyme (33-35); however, others report finding amounts of the M-type isozyme (36, 37). Furthermore, with increasing time of hepatocytes in culture, a shift to less L-type and more M-type has been reported (38-40). However, these previous experiments did not include insulin in the culture medium. In the experiments reported here, the L-type isozyme of pyruvate kinase comprised over 90% of the total enzyme activity in freshly isolated hepatocytes or intact liver. In hepatocytes cultured in the presence of insulin, the percentage of the L-type isozyme and pyruvate kinase catalytic activity remained constant throughout the culture period. These findings strongly emphasize the important role of insulin in the maintenance of L-type pyruvate kinase in hepatocytes.

Primary hepatocyte cultures have been used to investigate the effect of hormones such as insulin on the regulation and synthesis of several hepatic enzymes under well defined conditions. However, one problem commonly encountered in using cultured hepatocytes is the initial decrease in the catalytic activity of many of the specific enzymes being investigated with increasing time of culture. For instance, the glucokinase activity in hepatocytes (in the presence of insulin) decreased within 1 day in culture to values 10 to 25% of that measured in freshly isolated cells (41, 42). The glucokinase activity remained low for 3 days and then increased rapidly to levels observed in freshly isolated hepatocytes by day 6 and remained constant throughout the remaining culture period. A 2-fold increase in glucokinase activity does occur within 2 to 4 h after insulin addition (41). However, the approximately 10-fold increase of glucokinase primarily occurred over a 3-day period between day 3 and day 6 in culture (42).

In the data presented here, the presence of insulin was essential for the maintenance of a "normal" level of pyruvate kinase activity in primary cultures of hepatocytes. For instance, the level of pyruvate kinase in freshly isolated hepatocytes from a normal rat was maintained constant throughout the culture period if insulin was included in the culture medium. However, when the cells were cultured for even 24 h in the absence of insulin, the enzyme activity decreased. The time course for insulin induction of pyruvate kinase back to control values was rather similar to that observed with glucokinase (42). When hepatocytes were maintained in the absence of insulin for 24 h, the pyruvate kinase activity did not immediately return to "control" levels upon addition of insulin to the culture medium. Instead, the enzyme activity continued to decrease for 4 days. Then, the enzyme activity increased rapidly back to normal values over the next 3 days (Fig. 1). Likewise, when hepatocytes isolated from diabetic animals were cultured in medium containing insulin, a similar time course was observed with low levels of enzyme activity followed by a rapid induction between day 3 and day 7 to values which exceed control values (Fig. 3). This increase in the liver pyruvate kinase activity must result from an increase in the steady state level of pyruvate kinase protein in the cell. This could occur by an increase in the rate of synthesis of the enzyme or a decrease in the rate of catabolism. An increase in the relative rate of synthesis of liver pyruvate kinase (Fig. 5) preceded the increase in catalytic activity (Fig. 3) upon addition of insulin to hepatocytes isolated from a diabetic rat. Between days 7 and 10, the pyruvate kinase enzyme activity decreased back to around 14 enzyme units/ mg of DNA. This must result from a decrease in the steady state level of pyruvate kinase which could result from a decrease in the rate of synthesis, an increase in the catabolism, or both. As illustrated in Fig. 5B, the relative rate of liver pyruvate kinase synthesis was maximal between days 3 to 6 and seemed to be decreased slightly on day 7. Thus, around 2 days following the addition of insulin to the hepatocytes isolated from a diabetic rat, there was an increase in the relative rate of liver pyruvate kinase synthesis. This preceded an increase in the steady state level of the pyruvate kinase catalytic activity. The catalytic activity continues to increase over a 4-day period to a maximum level which exceeds normal or control values. When the normal values of enzyme activity are exceeded, some other intracellular control mechanism exerts its effect which results in a decreased rate of synthesis, increased rate of catabolism, or both. The result is a modulation of the total pyruvate kinase activity back to within the normal range of values.

Administration of insulin both in vivo and in vitro resulted in elevated amounts of liver pyruvate kinase synthesis. The more rapid response of liver pyruvate kinase catalytic activity in vivo than in vitro upon administration of insulin is probably a result of an altered endocrine status in vivo in addition to any direct effect of insulin on the pyruvate kinase activity in
The lag phase observed in vitro during hormone-stimulated enzyme induction remains unexplained. However, on the basis of previous reports, it is assumed that the lag phase may be a reflection of DNA-RNA synthesis preceding the translation to enzyme protein. Further studies are in progress to evaluate this assumption.


REFERENCES