Gluconeogenesis in Rabbit Liver

I. PYRUVATE-DERIVED DICARBOXYLIC ACIDS AND PHOSPHOENOLPYRUVATE FORMATION IN RABBIT LIVER*

(Received for publication, March 30, 1972)

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SUMMARY

Rabbit liver mitochondria incubated in a basic medium containing pyruvate, bicarbonate, MgSO₄, and P₁ synthesize and liberate to the medium malate, citrate, and also aspartate (provided NH₄⁺ is included in the medium). Only a minor amount of P-enolpyruvate is formed and liberated under these same conditions. Substrate utilization and especially metabolite production are dependent on exogenous P₁, and also quite dependent on exogenous bicarbonate. Exogenous ATP inhibits substrate utilization and especially inhibits total metabolite formation.

Mitochondria from 48-hour fasted rabbits or from fed alloxan diabetic rabbits as contrasted to those from normal fed rabbits appear to use more pyruvate and to synthesize significantly greater amounts of P-enolpyruvate and malate. The amounts of P-enolpyruvate formed, although larger, are still extremely small and considered insufficient to satisfy the rabbit's requirements for glucose.

Cytosol prepared from livers of normal fed rabbits contain a measurable amount of P-enolpyruvate carboxykinase; cytosol from livers of fasted or alloxan diabetic rabbits contain substantially greater quantities of this enzyme. Refeeding of fasted rabbits diminishes the cytosolic content of carboxykinase to near normal levels.

Distribution studies with cytochrome oxidase and citrate synthetase as marker enzymes confirm the in vivo existence of P-enolpyruvate carboxykinase in rabbit liver cytosol.

Cytosolic malate dehydrogenase, aspartate aminotransferase, and fumarate dehydratase activities, generally speaking, respond very little to alterations in nutritional states which substantially affect cytosolic carboxykinase.

These data support the concept that cytosolic P-enolpyruvate carboxykinase can contribute significantly to gluconeogenesis in this species.

* This work was supported by grants from the National Institutes of Health, AM 12705 and Training Grant 2T1-GM-565, and the American Diabetes Association. Parts of the data presented in this paper were taken from a dissertation submitted by D. C. J. and from theses submitted by R. A. B. and K. A. E. to the Graduate School of the University of North Dakota. A preliminary report has been presented before the Biological Section of the American Chemical Society (1).

†Supported by National Defense Fellowships.

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The pathway of hepatic glucose synthesis from pyruvate and its precursors has been extensively investigated in rat liver (2-4). An initial reaction involving carboxylation of pyruvate catalyzed by pyruvate carboxylase (EC 6.4.1.1) occurs predominantly in mitochondria (5, 6) whereas an obligatory succeeding reaction involving a decarboxylation and phosphorylation of oxalacetate catalyzed by P-enolpyruvate carboxykinase (EC 4.1.1.32) takes place mainly in cytosol (7). Mitochondrially formed oxalacetate does not appear to pass into the cytosol directly but is instead transferred in the form of malate, aspartate, or citrate to the cytosolic portion of the cell where malate and aspartate at least may serve as sources of carbon and reducing equivalents for glucose formation (2, 3, 8). Much evidence has accrued to support the participation of this shuttle system in glucose formation in the rat where P-enolpyruvate carboxykinase is essentially only in the cytosolic compartment of the cell.

On the other hand, relatively little attention has been given to the pathway of glucose formation in species such as the rabbit where P-enolpyruvate carboxykinase had, until recently, been reported to be present essentially only in hepatic mitochondria (7). Thus, in studies previously reported in which rabbit liver was used (9-11) it was assumed that P-enolpyruvate essential for glucose formation had to originate from mitochondria. These studies were reported subsequent to a report by Ilyin et al. (12) but prior to our preliminary communication (13) and that of Ustentoko (14); all of these studies indicated that P-enolpyruvate could be synthesized in rabbit liver cytosol in addition to mitochondria. These observations have recently been confirmed by Garber and Hanson (15) who, nonetheless, have concluded on the basis of their work with isolated mitochondria that synthesis of P-enolpyruvate for gluconeogenesis in rabbit liver occurs in both cytosol and mitochondria each functioning under different sets of controls.

In this report we present data to substantiate the recent suggestion of ourselves (13) and others (14, 15) that a sufficient quantity of P-enolpyruvate for glucose formation need not originate solely from the hepatic mitochondria of this species.

EXPERIMENTAL PROCEDURE

Young, male rabbits of the New Zealand strain weighing approximately 2 kg were obtained from Gopher State Caviary, St. Paul, Minn., and were maintained on Purina laboratory chow and tap water ad libitum unless experimental protocol required otherwise. Rabbits were made diabetic by a single injection of a
solution of 0.9% sodium chloride and recrystallized alloxan monohydrate (160 mg per kg body weight) into the marginal vein of the ear (16). Diabetic rabbits were used approximately 1 week after treatment with alloxan provided that their blood glucose levels were at least 350 mg/100 ml. The condition of diabetes was further verified by histological examination which showed degeneration of the B-cells of the pancreas of rabbits treated with alloxan.

Blood glucose was determined by the glucose oxidase method (Glucostat, Worthington Biochemical Corp.). Protein concentrations were determined by the biuret method (17), as modified by Lowry and Larson for membrane-bound systems (18).

Rabbits were killed by a blow on the head followed by decapitation. Twice washed hepatic mitochondria prepared by the method of Schneider (19) were resuspended in 0.25 m sucrose and used immediately in the incubation experiments. Mitochondrial preparations were periodically checked on the Gilson Oxygraph to establish that they exhibited normal characteristics of oxygen uptake which could be inhibited by addition of ATP (20). Cytosolic fractions were obtained by centrifugation at 105,000 × g for 1 h, the microsomal pellet subsequently being washed and reprecipitated by centrifugation.

Incubations were carried out in stoppered 25-ml Erlenmeyer flasks, shaken in a water bath maintained at 37° with liver mitochondria prepared from fed rabbits unless stated otherwise.

Each flask contained a basic medium of 6.7 mM potassium phosphate, pH 7.4; 6.7 mM triethanolamine, pH 7.4; 7.5 mM MgSO4; 6.7 mM sodium pyruvate; and 10.0 mM KHCO3 made up to a volume of 2.5 ml with isotonic sucrose (2, 3). Other additions were made as described in the various figures and tables. After a 2-min preincubation period, the reactions were initiated by the addition of 0.5 ml (5 to 7 mg of protein) of mitochondrial suspension and, after 10 min, were terminated by placing the flasks in ice. The mitochondria were separated from the reaction media by centrifugation, and the resulting supernatant solutions plus mitochondrial washes were acidified with 3 ml of 6% HClO4. The KClO4 precipitate was removed by filtration and the samples were stored at -20° until the metabolites were assayed. Pyruvate, P-enolpyruvate, aspartate, malate, and a-ketoglutarate were assayed according to procedures described by Bergmeyer (21). Citrate was measured by the method of Moellering and Gruber (22).

Aliquots of mitochondrial preparations were assayed for their content of substrate and metabolites both before and after in cubation to ensure that they did not initially contain, nor subsequent to the incubation period retain, significant amounts of these compounds. In both cases, the amounts of substrate and metabolites were found to be negligible relative to the amounts of added substrate and metabolites formed in and liberated from the mitochondria.

For enzyme studies, mitochondrial preparations were frozen and thawed three times prior to being assayed for enzyme activities. All studies were performed on mitochondrial, cytosolic, and microsomal fractions freshly thawed after being stored at -20° from the time of preparation.

P-enolpyruvate carboxykinase (EC 4.1.1.32) was assayed at 30° by the method of Nordlie and Lardy (7) as modified by Foster et al. (23). All other assays were performed on a Beckman (model DK-2) or on a Bausch and Lomb (model 505) Spectrophotometer at 25° according to the following procedures: cytochrome oxidase (EC 1.9.3.1.C), Smith (24); citrate synthetase (EC 4.1.3.7.C), Srere (25); malate dehydrogenase (EC 1.1.1.37), Ochoa (26); aspartate aminotransferase (EC 2.6.1.1), Karmen (27); and fumarate hydratase (EC 4.2.1.2C), Racker (28).

RESULTS

Establishment of Basic Incubation Conditions—Although the basic incubation medium finally employed was, except for the omission of ATP, the same as that used by Lardy et al. (2), we measured the effects of altering various experimental conditions on substrate utilization and metabolite production to assure that these conditions were near-optimum; the data obtained are presented in Fig. 1.

The ability of isolated mitochondria to convert pyruvate primarily into malate, citrate, and, to a much lesser extent, P-enolpyruvate is depicted in Fig. 1A. Optimal amounts of these metabolites were produced with a pyruvate concentration of 6.7 mM. Net formation of these metabolites from pyruvate is necessarily dependent on carboxylation; optimal substrate utilization and metabolite production was achieved with 10 mM KHCO3 (Fig. 1B). Utilization of substrate and the production of metabolites were, except in the case of malate production, essentially linear over a 25-min period (Fig. 1C). Nonetheless, the decreasing rate of malate production led us to limit our incubation period to 10 min.

Since metabolic production was found to be nearly totally dependent on the presence of Pi, 6.7 mM Pi was included in the medium. Inclusion of 7.5 mM MgSO4 was found to markedly enhance both substrate utilization and metabolite production to optimal levels compared to the very low levels of both in its absence.

Basal Production of Metabolites and Influence of NH4Cl—Comparison was made of pyruvate utilization and the production of P-enolpyruvate, malate, citrate, and aspartate by mitochondria incubated in basic medium alone or with 3 mM NH4Cl added (Fig. 2). Addition of NH4Cl to the basic medium resulted in aspartate becoming the major metabolite apparently at the expense of citrate and especially malate which are the two major metabolites produced by incubation of pyruvate in the basic medium alone. Production of P-enolpyruvate is similar in both cases and at best represents only a small contribution to the total amount of metabolites produced.

Lack of Requirement for Exogenous ATP—Pyruvate carboxylase is known to require ATP as a cofactor (5). However in this system, increasing the concentration of ATP in increments from 0 to 6 mM in general decreases pyruvate utilization and metabolite production (Fig. 3). Although P-enolpyruvate production is effected the least and in fact increases slightly with addition of ATP at concentrations of 0.75 and 1.5 mM, it decreases substantially with 3 and 6 mM ATP. These observations were reproducibly reproducible with ATP from various sources and also in the presence of an ATP-generating system (creatine phosphate-creatine kinase).

Effects of Adding Other Nucleotides—Although GTP is the in vivo cofactor for P-enolpyruvate carboxykinase (29), its inclusion in the incubation media exerts essentially no effect on substrate utilization and metabolite formation (Fig. 3), perhaps due to an inability to enter mitochondria.

Since ITP can replace GTP as a cofactor for P-enolpyruvate carboxykinase in vitro (29), its effect on the system was also investigated. We find as much P-enolpyruvate carboxykinase activity liberated by three freeze-thawings as by sonication with a Bronwill Biosonic sonicator.
Fig. 1. The effects of altering pyruvate (A); KHCO₃ (B); and time on pyruvate utilization or metabolite production, or both (C). Other incubation conditions (except for the one varied) are similar to those described under "Experimental Procedure." The results depicted are representative of repeated observations. Symbols used are: ○, pyruvate; □, citrate; Δ, malate; ◆, P-enolpyruvate.

Fig. 2. The effects of 3 mM NH₄Cl on pyruvate utilization and metabolite production. Other incubation conditions are similar to those described under "Experimental Procedure." The results depicted are averages of data from six experiments and the vertical lines represent standard deviations. Symbols used are: ■, basic reaction medium; □, basic reaction medium plus 3 mM NH₄Cl; PYR, pyruvate; PEP, P-enolpyruvate; MAL, malate; CIT, citrate; ASP, aspartate.

Investigated. It is apparent from Fig. 3 that ITP decreases substrate utilization nearly as much as does ATP. In addition, it depresses P-enolpyruvate formation to a greater extent while depressing citrate and especially malate formation to a lesser extent than does ATP. Of the various nucleotides tested in the system, 1 mM ADP causes the greatest decrease in pyruvate utilization and in metabolite formation (Fig. 3) and, unlike ATP, decreases P-enolpyruvate formation as well. These observations made with the different nucleotides were qualitatively quite reproducible with various preparations of mitochondria.

Influence of α-Ketoglutarate or Glutamate on Pyruvate Utilization

As concentrations of ADP were decreased from 1.0 to 0.5 and 0.1 mM, the suppression of pyruvate utilization and metabolite production was substantially relieved; nonetheless 0.5 mM ADP still exerted very obvious deleterious effects. In the presence of 1 mM ADP utilization of pyruvate and production of P-enolpyruvate and citrate were suppressed but were linear over a 10 min time period. Production of malate was negligible however.
Fig. 4. The effects of 3 mM \( \alpha \)-ketoglutarate (■) or 3 mM glutamate (▲) on pyruvate (PYR) utilization and metabolite production. Other incubation conditions are similar to those described under "Experimental Procedure." Aspartate (ASP) was not measured in incubations carried out in the presence of \( \alpha \)-ketoglutarate. Values represent averages of data from five experiments. The vertical lines represent standard deviations. Significance of differences between sample means; data from incubations of pyruvate in the presence of \( \alpha \)-ketoglutarate or glutamate tested against data obtained with pyruvate alone. PEP, P-enolpyruvate; MAL, malate; CIT, citrate; ASP, aspartate.

and Metabolite Production—In order to further evaluate the influence of substrate level phosphorylation and GTP production on P-enolpyruvate formation, we tested the effect of adding 3 mM \( \alpha \)-ketoglutarate to the system and these data are presented in Fig. 4.

Addition of \( \alpha \)-ketoglutarate significantly decreases pyruvate utilization and significantly increases the production of citrate. While the increase in P-enolpyruvate production was consistently somewhat higher in nearly all experiments when pyruvate was incubated with \( \alpha \)-ketoglutarate, the increase was not statistically significant. Malate production was usually lower under the same conditions but again, a statistically significant difference in malate production was not observed.

The ability of 3 mM glutamate to effect pyruvate utilization and metabolite production was also evaluated (Fig. 4). Addition of glutamate causes a statistically significant decrease in malate production concomitant to causing a significant increase in aspartate production. Again, P-enolpyruvate production was consistently increased above the control level, but the average increase is only of marginal significance.

Effects of Fasting and Diabetes on Mitochondrial Substrate Utilization and Metabolite Production—Since both fasting and diabetes are known to enhance the rate of gluconeogenesis, we compared substrate utilization and metabolite production by mitochondria isolated from 48-hour fasted, normal, fed, alloxan diabetic rabbits and fed control rabbits; these data are presented in Fig. 5.

5 in terms of substrate utilization and metabolite production g of liver \(^{-1} \) 10 min \(^{-1} \). Fasting, in general, appears to cause increases in both pyruvate utilization and metabolite production although only the increase in malate formation is significant \((p < 0.025)\). Mitochondria from fed, diabetic rabbits also appear to use more pyruvate and form significantly greater amounts of both malate \((p < 0.01)\) and P-enolpyruvate \((p < 0.02)\). Changes in hepatic glycogen and lipid content known to occur during conditions such as fasting or diabetes could influence data expressed on the basis of liver weight. Nonetheless the differences in metabolite production still obtain with approximately the same or slightly greater degrees of statistical significance when these same data are expressed on the basis of either mitochondrial protein content or per 100 g of body weight.

Production of P-enolpyruvate from Aspartate and Malate by Rabbit Liver Cytosol—Although mitochondria incubated with pyruvate produce little P-enolpyruvate under the conditions tested, substantial amounts of both aspartate and malate were produced and liberated into the medium. Therefore, we examined the capacity of rabbit liver cytosol to convert aspartate and malate into P-enolpyruvate according to procedures described by Shrago and Lardy (8).

Cytosol prepared from livers of fed or fasted rabbits were incubated under the assay conditions normally employed for P-enolpyruvate carboxykinase replacing the normal substrate, oxalacetate, with either aspartate and \( \alpha \)-ketoglutarate or with malate, DPN, and pyruvate. Data indicated that liver cytosol from fed rabbits can convert either aspartate or malate into P-enolpyruvate and that fasting greatly enhances this cytosolic capacity for P-enolpyruvate formation from either substrate. These data provided a preliminary indication that rabbit liver cytosol contained P-enolpyruvate carboxykinase as well as malate dehydrogenase and aspartate aminotransferase activity.
confirmed that the carboxykinase activity in cytosol from rabbits was not an artifact of mitochondrial origin liberated during preparative procedures. Thus we assayed mitochondrial, cytosolic and microsomal fractions for activities of the exclusively mitochondrial enzymes, citrate synthetase and cytochrome oxidase (30), and these data are presented in Table II.6

Data presented indicate that cytosolic and microsomal fractions from livers of fed rabbits contain approximately 10% of the total citrate synthetase activity measured in the three subcellular fractions. Cytosolic plus microsomal fractions from livers of fasted and diabetic rabbits contain about 15% of the total citrate synthetase activity. Thus a minimum of 85 to 90% of the activity which should be associated with mitochondria is found in that fraction and there appears to be only a slight increase in the loss of mitochondrial contents into the cytosolic and microsomal fractions from livers of fasted or diabetic rabbits.

Results quite similar to those presented for citrate synthetase also obtain when cytochrome oxidase is used as the indicator of presence of P-enolpyruvate carboxykinase in rabbit liver cytosol although we were not aware of their data until our own work was underway. In addition no workers had offered any definitive data to support the in vivo existence of P-enolpyruvate carboxykinase in rabbit liver until the recent studies of Garber and Hanson (15).

* Data in Table II were collected from rabbits which are also included in the larger groups of animals for which P-enolpyruvate carboxykinase data are reported (Table I).

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### Table I

<table>
<thead>
<tr>
<th>Condition</th>
<th>Mitochondrial activity</th>
<th>Cytosolic activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Specific activity</td>
<td>Total activity</td>
</tr>
<tr>
<td>Fed (10)</td>
<td>39.5 ± 19</td>
<td>77.7 ± 39</td>
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<tr>
<td>Fasted, 48 hrs (15)</td>
<td>78.7 ± 24</td>
<td>58.9 ± 33</td>
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<tr>
<td>Fed, diabetic (11)</td>
<td>93.2 ± 23</td>
<td>116 ± 58</td>
</tr>
<tr>
<td>Fasted, 48 hrs, plus refeeding (12)</td>
<td>63.7 ± 13</td>
<td>127 ± 28</td>
</tr>
</tbody>
</table>

a Specific activity in nanomoles of P-enolpyruvate formed min⁻¹ mg of protein⁻¹.
b Total activity in micromoles of P-enolpyruvate formed min⁻¹ total hepatic subcellular fraction⁻¹.
c Number of rabbits.
d Standard deviation from the mean.
e Significance of difference between sample means; data from fasted, fed diabetic and fasted, refed rabbits tested against data from fed controls.

### Table II

<table>
<thead>
<tr>
<th>Condition</th>
<th>Total activity in subcellular fractions</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. Citrate synthetase</td>
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</tr>
<tr>
<td>Fed (12)</td>
<td>106 ± 31</td>
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<tr>
<td>Percentage of total hepatic activity</td>
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<tr>
<td>Fasted, 48 hrs (6)</td>
<td>78.1 ± 32</td>
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<tr>
<td>Percentage of total hepatic activity</td>
<td>89.7%</td>
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<tr>
<td>Fed diabetics (6)</td>
<td>85.2 ± 27</td>
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<tr>
<td>Percentage of total hepatic activity</td>
<td>83.6%</td>
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<tr>
<td>B. Cytochrome oxidase</td>
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<tr>
<td>Fed (11)</td>
<td>322 ± 145</td>
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<tr>
<td>Percentage of total hepatic activity</td>
<td>93.3%</td>
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<tr>
<td>Fasted, 48 hrs (6)</td>
<td>140 ± 107</td>
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<tr>
<td>Percentage of total hepatic activity</td>
<td>85%</td>
</tr>
<tr>
<td>Fed diabetics (6)</td>
<td>162 ± 116</td>
</tr>
<tr>
<td>Percentage of total hepatic activity</td>
<td>78.2%</td>
</tr>
</tbody>
</table>

a Total activity expressed in micromoles of product formed min⁻¹ total hepatic subcellular fraction⁻¹ and represents the percentage of total activity found in the three fractions rather than that of a total homogenate.
b Number of rabbits.
c Standard deviation from the mean.

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Intracellular Location of P-enolpyruvate Carboxykinase—Data pertaining to the intracellular distribution and activity of P-enolpyruvate carboxykinase in rabbit liver are presented in Table I. In the fed rabbit, the majority of the enzyme is located in the mitochondrial fraction of the cell as previously reported by Nordlie and Lardy (7), Ilyin et al. (12), and more recently by ourselves (13) and others (14, 15). Although the data in the table have not been corrected for loss of mitochondrial contents into the nuclear pellet, or into cytosol due to mitochondrial damage or lability (see Table II), the P-enolpyruvate carboxykinase in mitochondria is, in terms of specific activity, still approximately 8-fold higher than that of the enzyme located in cytosol. Furthermore, the cytosol appears to contain only about 10% (when corrected for mitochondrial loss and cytosolic contamination) of the total hepatic carboxykinase activity.

Effects of Fasting and Diabetes on P-enolpyruvate Carboxykinase Activity—The conditions of fasting and alloxan diabetes cause highly significant increases in the specific activities of carboxykinase in both mitochondrial and cytosolic portions of the cell (Table I). However, fasting and diabetes also cause markedly significant increases of 4- to 6-fold (5- to 7-fold corrected) in the total activity of the cytosolic enzyme while no significant changes appear in the total activity contained within the mitochondria. Additionally, refeeding 48-hour fasted animals for 48 hours returns the specific and total activities of the cytosolic enzyme to near control levels.

Confirmation of Intracellular Location of P-enolpyruvate Carboxykinase—Since P-enolpyruvate carboxykinase had, up until the time of our preliminary communication (13) been reported to be a mitochondrial enzyme (to our knowledge⁵), it was necessary to...
cytosolic contamination with mitochondrial contents (Table II). Approximately 7, 15, and 20% of the total cytochrome oxidase activity is found in the cytosolic plus microsomal fractions prepared from livers of fed, fasted, and diabetic rabbits, respectively.

Activities of Other Enzymes in Hepatic Cytosol from Rabbits
Since P-enolpyruvate carboxykinase is located in both mitochondria and cytosol of rabbit liver and since its cytosolic activity is increased under conditions which cause an increase in glucose formation, we concluded that gluconeogenesis in rabbits probably occurs at least in part according to processes similar to those thought to occur in rat liver (13). If so, then aspartate aminotransferase, malate dehydrogenase, and fumarate hydratase would all be involved in the conversion of cytosolic malate and aspartate to oxaloacetate which could subsequently serve as substrate for cytosolic P-enolpyruvate carboxykinase. Accordingly we assayed the activities of these enzymes in hepatic cytosols prepared from rabbits subjected to conditions previously found to alter cytosolic carboxykinase activity.

Cytosolic activities of malate dehydrogenase, aspartate aminotransferase, and fumarate hydratase were found to be 8130, 845, and 1736 μmoles of product formed min⁻¹ total liver⁻¹. Our data indicate that fasting causes relatively insignificant decreases in the activities of malate dehydrogenase and fumarate hydratase. Refeeding 48-hour fasted rabbits results in activity levels of all three enzymes unchanged from normal values. Diabetes as contrasted to fasting results in an extensive and significant increase in the activity of aspartate aminotransferase although the total activities of malate dehydrogenase and of fumarate hydratase remain unaffected.

DISCUSSION
The reported location of P-enolpyruvate carboxykinase in rabbit liver (7) provided the initial impetus for our studies in light of the following considerations. If P-enolpyruvate carboxykinase were solely a mitochondrial enzyme, then P-enolpyruvate would have to be formed in and liberated from the mitochondria if gluconeogenesis were to follow the accepted pathway for glucose formation. A mitochondrial source of reducing equivalents would also be needed for carbohydrate formation from certain substrates (alanine for example).

The ability of isolated rat liver mitochondria to affect a net synthesis of malate, aspartate, and citrate from pyruvate has been well documented (2-4). Such mitochondria, from which P-enolpyruvate carboxykinase is virtually absent (7, 31) do not make and liberate detectable amounts of P-enolpyruvate from pyruvate (2, 3) although they can produce P-enolpyruvate under certain conditions given the proper substrate (32).

Rabbit liver mitochondria have been shown by Davis and Gibson (10, 11) to produce malate, citrate, and P-enolpyruvate from pyruvate. The amount of P-enolpyruvate produced by their system is quite small relative to the production of other metabolites and is, quite similar to the amount of P-enolpyruvate produced by our system. However, the amount of pyruvate converted to P-enolpyruvate by their system is extensively increased, up to a point by adding increasing amounts of uncouplers such as DNP or oleate. The physiological significance of these observations with uncouplers may be questionable considering the requirement for ATP in pyruvate carboxylation and also in the over-all synthesis of carbohydrate.

Our experiments confirm some of the observations of Davis and Gibson (10, 11). We observe a net synthesis of malate, citrate, and P-enolpyruvate with our system (Figs. 1 and 2). In addition, we have demonstrated the net synthesis of aspartate from pyruvate when NH₄⁺ is included in the media (Fig. 2).

There is an apparent lack of a requirement, by this system, for exogenous ATP (Fig. 3) although CO₂ fixation is known to be ATP-dependent (5). Krebs et al. (33) have shown that the addition of ATP reduces the formation of glucose by pigeon liver homogenates. In contrast Garber and Ballari (34) found substantial rates of P-enolpyruvate production by guinea pig liver mitochondria from certain Krebs’ cycle intermediates only in the presence of added ADP or ATP but formation of P-enolpyruvate from pyruvate was also low under their conditions. Nonetheless, a substantial net production of metabolites from pyruvate by our system (Fig. 2) suggests that endogenous production of ATP must be sufficient to support CO₂ fixation.

The relative lack of P-enolpyruvate formation by our system suggests an inability of mitochondrial P-enolpyruvate carboxykinase to compete effectively for oxaloacetate. If adequate endogenous ATP is available, then the lack of P-enolpyruvate formation from pyruvate may simply reflect the low nucleoside diphosphokinase activity in rabbit liver mitochondria (15, 35). On the other hand endogenous production of ATP may not be adequate to permit the formation of sufficient amounts of GTP. Although exogenous GTP exerts no effect (Fig. 3) most likely due to an inability to enter the mitochondria (36), ITP which apparently does enter the mitochondria (judged by its effect on the system (Fig. 3)) also does not enhance P-enolpyruvate production. Furthermore while inclusion of either α-ketoglutarate or glutamate with pyruvate does increase P-enolpyruvate production somewhat the increase is not significant, especially considered in relationship to the amounts of other metabolites (Fig. 4). Thus the availability of a substrate capable of providing GTP via substrate level phosphorylation does not substantially enhance the production of P-enolpyruvate from pyruvate by our system.

Addition of low levels of ATP does appear to increase P-enolpyruvate production somewhat while citrate and especially malate formation is lowered. If the amounts of ATP added do not suppress oxidation but support pyruvate carboxylation and provide an increased level of GTP (via nucleoside diphosphokinase), the increase in P-enolpyruvate formation along with the decrease in the net production of other metabolites might be accounted for.

The ability of higher concentrations of ATP to suppress pyruvate utilization and metabolite production remains unexplained. While respiration is inhibited by ATP in tightly coupled systems (20), this should not alter pyruvate carboxylation unless levels of acetyl-CoA become limiting. This would seem unlikely since sufficient acetyl-CoA is available to account for considerable citrate formation compared to the amount of malate formed. Exogenous ADP exerts more drastic effects on pyruvate utilization and metabolite production than does ATP (Fig. 3) perhaps, according to the findings of Walter et al. (3), by inhibition of pyruvate carboxylation although such an effect by ADP has been questioned (11). While it was considered possible that exogenous ATP was contaminated with ADP or was giving rise to ATP via ATPase, the effects of exogenous ATP were not eliminated by including an ATP-generating system in the incubation mixture thus presumably eliminating these possibilities. Additionally, qualitatively similar effects were also observed with ITP.

Mitochondria from fasted rabbits and especially from diabetic rabbits appear to use more pyruvate and to produce significantly larger amounts of malate and P-enolpyruvate (Fig. 5). We can...
presently offer no explanation for these observations but two possibilities are (a) enhanced pyruvate carboxylation activity and (b) a greater uptake of substrate and there are data to support both. Pyruvate carboxylase activity is reportedly increased in rat and mouse liver mitochondria by both fasting and diabetes (37). Nonetheless, recent data of Garber and Hanson (15) and our own unpublished data suggest that such is not the case in rabbit liver. Adam and Haynes (38) have shown that injection of cortisol, which promotes gluconeogenesis, increases the rate of pyruvate uptake by rat liver mitochondria and this effect apparently precedes activation of pyruvate carboxylase. Such an effect has not been ruled out here.

Although production of P-enolpyruvate by rabbit liver mitochondria appears to be increased by fasting and diabetes (Fig. 5), its production is still extremely small relative to the total amount of metabolites formed. Data of Solomon et al. (39) suggest that the rate of P-enolpyruvate formation needed to satisfy a fasting rat's requirements for glucose is approximately 1 amole min⁻¹ g of liver⁻¹. We have calculated a rate of about 0.7 amole of P-enolpyruvate min⁻¹ g of liver⁻¹ formed during glucose formation from lactate by perfused livers isolated from fasted rabbits, suggesting somewhat similar minimal requirements in the two species. Such a rate of P-enolpyruvate formation from pyruvate would require reducing equivalents sufficient for conversion of that P-enolpyruvate to triose phosphate.

We observe 20 to 25% of the required amount of reducing equivalents released to cytosol in the form of malate with mitochondria from fasted or diabetic rabbits. Similar or even larger amounts are probably released to the medium as malate and aspartate when NH₄⁺ is added although production of aspartate by mitochondria from fasted or diabetic rabbits was not measured. This amount of reducing equivalents is a minimum figure since it is calculated only on the basis of mitochondria isolated and also does not include fumarate which is almost certainly formed in the system (3). Thus it seems that reducing equivalents in the form of malate can be shuttled from mitochondria to cytosol in rabbit liver just as in rat liver. Aspartate could also contribute reducing equivalents if it were converted to oxaloacetate through the urea cycle-fumarase-malate dehydrogenase pathway.

The total amount of carbon liberated as malate, citrate, and P-enolpyruvate by the mitochondria from fasted or diabetic rabbits is approximately 35 to 40% of the assumed requirement, again calculated on the basis of the mitochondria isolated. Again, we assume that formation of aspartate in the presence of NH₄⁺ would not drastically alter the total amount of carbon liberated. Mitochondrially produced P-enolpyruvate released totals only about 5% of the required amount of carbon. Even the amount of P-enolpyruvate formed from pyruvate in the presence of uncouplers seems to fall short of the assumed requirements (10, 11).

Although our preliminary data suggest that rabbit liver cytosol has sufficient malate dehydrogenase, aspartate aminotransferase, and P-enolpyruvate carboxykinase to convert substantial amounts of malate and aspartate to P-enolpyruvate, the activity of citrate cleavage enzyme is extremely low and probably insignificant to the gluconeogenic process.

Data previously reported by us (13), others (12, 14, 15), and also in Table I, show that a measurable amount of P-enolpyruvate carboxykinase can be found in the cytosolic fraction of normal rabbit liver although most of the activity is located in the mitochondria as first reported (7). However, the cytosolic activity responds during fasting and diabetes to the point that total hepatic P-enolpyruvate carboxykinase activity in cytosol is increased 5- to 6-fold (Table I). Our observations agree with those of Ilyin et al. (12), Usatenko (14), and Garber and Hanson (15).

Additional circumstantial evidence for the involvement of cytosolic carboxykinase in gluconeogenesis is provided by Ilyin et al. (12) who observed that prolonged treatment (54 hours) with hydrocortisone increases cytosolic carboxykinase activity. We have found in preliminary studies that treatment of fed rabbits with hydrocortisone for 12 to 16 hours or with mannose for only 4 hours causes significant increases in cytosolic carboxykinase activity. Thus the cytosolic carboxykinase activity can, under certain circumstances, respond rapidly to conditions which elicit increased glucose synthesis.

Data related to cellular distribution of P-enolpyruvate carboxykinase (Table II) confirm those of Garber and Hanson (15) that this activity is present in rabbit liver cytosol in vivo. Carboxykinase activity is present in cytosols of fasted and diabetic rabbits in amounts sufficient to produce twice the amount of P-enolpyruvate that may be required for minimal glucose formation.

Garber and Hanson (15) have recently shown that rabbit liver mitochondria given α-ketoglutarate generate malate, citrate, and lesser amounts of P-enolpyruvate. Additionally they find that as the NADH:NAD⁺ ratio is increased toward that observed in livers of fasted rabbits, the amount of mitochondrially produced P-enolpyruvate increases while that of malate and citrate decreases. Such findings present an anomaly considering that gluconeogenesis from many substrates requires cytosolic reducing equivalents and that cytosolic carboxykinase activity is extensively increased under such conditions.

On the basis of (a) the amount of P-enolpyruvate carboxykinase in hepatic cytosol of fasted and diabetic rabbits, (b) the amount of pyruvate-derived carbon which can be made available to the cytosol in the form of malate, citrate, or aspartate, or both, and (c) the high activities of auxiliary cytosolic enzymes needed for the conversion of these substrates to P-enolpyruvate, it seems reasonable to project that cytosolic carboxykinase most likely contributes significantly to gluconeogenesis in this species. Nonetheless the gamut of observations made in the studies of Garber and Hanson (15) and those reported here indicate that very complex control mechanisms must be involved as P-enolpyruvate production is shifted between mitochondria and cytosol.

REFERENCES

32. Granger, M., and Harris, E. J. (1971) Bioenergetics 2, 151-161
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