Mitochondrial Phosphoenolpyruvate Carboxykinase (GTP) and the Regulation of Gluconeogenesis and Ketogenesis in Avian Liver*

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Chicken liver synthesizes glucose from lactate and dihydroxyacetone at high rates but pyruvate, glycerol, alanine, and other amino acids are poor glucose precursors. Despite its limited conversion to glucose, 1 mm pyruvate completely suppressed ketone body synthesis from octanoate by perfused chicken liver, whereas lactate at this concentration had no effect on ketogenesis. Also, increasing the lactate concentration over a range of 0.5 to 10 mm caused a marked decrease in ketone body formation. In hepatocytes from chicken liver, pyruvate (10 mm) stimulated the oxidation of [1-14C]octanoate to 14CO2 5-fold, while decreasing net ketone body synthesis. Conversely, octanoate had no effect on the oxidation of [1-14C]pyruvate or [2-14C]pyruvate to 14CO2. The regulation of pyruvate metabolism by chicken liver mitochondria differed markedly from guinea pig liver mitochondria. The conversion of [2-14C]pyruvate to either [14C]acetoacetate or 14CO2 by isolated chicken liver mitochondria was not inhibited by octanoate oxidation to the same extent as was noted with mitochondria from guinea pig liver. Also, the decarboxylation of [1-14C]pyruvate to 14CO2 by guinea pig liver mitochondria was more sensitive to inhibition by octanoate. Our results suggest that the pyruvate dehydrogenase complex in chicken liver is not as sensitive to the inhibitory effects of fatty acid oxidation as is the same enzyme complex in guinea pig liver.

Measurement of intermediates in the pathway of gluconeogenesis from lactate, pyruvate, glycerol, and dihydroxyacetone was used to assess the points in the pathway where intermediates accumulate, especially after the simultaneous infusion of octanoate. With both lactate and pyruvate, octanoate infusion greatly increased the levels of citrate, α-ketoglutarate, and malate, with only a marginal effect on glycolytic intermediates, except for a decrease in the concentration of phosphoenolpyruvate. The most notable change was a 10-fold increase in oxalacetate concentration in chicken liver after perfusion with pyruvate and octanoate. The limitation in disposal of cytosolic NADH in chicken liver was apparent in experiments in which glycerol or glycerol plus octanoate were perfused simultaneously. In this experiment the concentration of α-glycerophosphate in the liver was greater than 5 μmol/g. Thus, the mitochondrial location of P-enolpyruvate carboxykinase is critical to the regulation of gluconeogenesis and ketogenesis in this species.

Gluconeogenesis in the rat has been extensively studied and the role of the cytosolic form of phosphoenolpyruvate carboxykinase (GTP) (EC 4.1.1.32) is well documented (1). Avian species such as the pigeon and the chicken offer an opportunity to examine the metabolic consequences of the mitochondrial location of the enzyme and to determine its impact on gluconeogenesis and ketosis. The usefulness of these species models in understanding the regulation of gluconeogenesis is readily apparent from several studies in which specific predictions concerning control of the pathway were verified. For example, pyruvate conversion to glucose in pigeon liver is limited by the fact that the intramitochondrial location of P-enolpyruvate carboxykinase precludes the well studied metabolic shuttle present in rat liver, in which malate movement from mitochondria to cytosol provides both the NADH required for glucose synthesis as well as cytosolic oxalacetate. In avian liver this oxalacetate cannot be further metabolized due to the absence of a cytosolic form of P-enolpyruvate carboxykinase, thus greatly limiting gluconeogenesis from pyruvate (2, 3).

A number of studies with the chicken have demonstrated that hepatic glucose synthesis occurs predominantly from lactate (4–7). Other gluconeogenic precursors important in mammals, such as glycerol, alanine, and pyruvate are converted to glucose at marginal rates by chicken liver (4–7). In a recent paper (4), we have shown that chicken kidney can synthesize glucose at appreciable rates from these precursors. Also this tissue (but not liver) contains an inducible cytosolic form of P-enolpyruvate carboxykinase which is immunochemically distinct from the mitochondrial form of the enzyme (4).

Based on these findings we have proposed that in species which contain both forms of the enzyme, the mitochondrial P-enolpyruvate carboxykinase is used specifically for lactate gluconeogenesis whereas the cytosol enzyme is important for gluconeogenesis from alanine, pyruvate, and other precursors.

In the present report we extend these studies to demonstrate limitations in hepatic ketogenesis in the chicken. Also, in birds the flux of pyruvate carbon through the hepatic pyruvate dehydrogenase complex is not as sensitive to the oxidation of fatty acids as is noted in mammalian liver.

EXPERIMENTAL PROCEDURES

Materials—Lactate dehydrogenase (EC 1.1.1.27), β-hydroxybutyrate dehydrogenase (EC 1.1.1.30), pyruvate kinase (EC 2.7.1.40), NAD, malate dehydrogenase (EC 1.1.1.37), glucose-6-phosphate dehydrogenase (EC 1.1.1.49), glutamate dehydrogenase (EC 1.4.1.3) Glycerol-3-phosphate dehydrogenase (EC 1.1.1.8), hexokinase (EC 2.7.1.1), fructose-biphosphate aldolase (EC 4.1.2.13), triosephosphate isomerase (EC 5.3.1.1), adenylyl kinase (EC 2.7.4.3), ATP, ADP, AMP, NAD, NADH, and sodium pyruvate were purchased from Boehringer.

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1 The abbreviation used is: P-enolpyruvate, phosphoenolpyruvate.
ger Mannheim, citrate (pro-38)-lyase (EC 4.1.3.6), L(-)-lactate, glutathione, glyceraldehyde, a-ketoglutarate, dihydroyxacetone, alanine and octanoate were from Sigma. All other chemicals were of the highest purity available and were purchased from Fisher.

**Animals**—Male Leghorn chickens (4-6 weeks old) weighing 250-400 g were used in these studies. The animals were starved for 48 h prior to use. Male guinea pigs (200-250 g) of the Hartley strain were fed a diet of Purina guinea pig chow and starved 48-72 h before use.

**Isolation and Incubation of Mitochondria**—Livers were removed from the animals, minced, and homogenized in a 5 mM 4-morpholinepropanesulfonic acid buffer, pH 7.4, containing 220 mM mannitol and 70 mM sucrose. The homogenate was diluted to 10% with buffer containing EDTA to a final concentration of 2 mM. After separation of nuclei and cell debris by centrifugation at 400 x g for 10 min, the mitochondria were sedimented at 7000 x g for 10 min. The mitochondrial pellet was washed twice and then resuspended in the buffer described above, to a final protein concentration of about 50 mg/ml.

Prior to using these mitochondria the respiratory control ratio was determined in thermostated cuvettes using a Clark-type oxygen electrode. The average respiratory control ratio measured in control incubations of mitochondria from both chicken and guinea pig liver was about 8.0. Mitochondrial protein concentration was measured by a biuret procedure using bovine serum albumin as a standard (8).

Approximately 12 mg of mitochondria were incubated in 5 mM 4-morpholinepropanesulfonic acid buffer, pH 7.4, containing 220 mM mannitol, 70 mM sucrose, 3.3 mM ADP, and when added 2 mM pyruvate and 0.2 mM octanoate. The total volume was 3 ml and incubation was carried out at 38°C for 10 min. In these experiments we determined the metabolic interaction between pyruvate and octanoate by incubating mitochondria with 2 mM [1-14C]pyruvate (2-14C)pyruvate (0.5 μCi) containing unlabeled octanoate or 0.2 mM [1-14C]octanoate (0.5 μCi) containing unlabeled pyruvate. The incubation was terminated by the addition of perchloric acid to a final concentration of 6%. The 14CO2 was trapped and counted as described previously (9).

The [14C]acetoacetate formed was isolated by a modification of the method of Werchselbaum and Smygot (10). In this procedure the incubation medium was neutralized for the enzyme determination of acetoacetate (11); a portion of this neutralized extract was distilled in 100 ml of H2O, containing 3 ml of 50% H2SO4 and 20 μmol of carrier 4-acetoacetate. The acetoacetate, which is decarboxylated by heating in acid is converted to acetone which is then trapped in 6 ml of HgSO4 (in 3 ml of H2SO4) as the mercury salt. This salt was solubilized in 0.5 ml of 4 N HCl and the radioactivity determined as described by McCarty et al. (12). Recovery of unlabeled acetone was 80-90%. Since β-hydroxybutyrate synthesis by both chicken and guinea pig liver mitochondria is less than 30% of total ketone body synthesis (13), only the specific activity of acetoacetate was measured.

**Liver Perfusion**—Chickens starved for 48 h were anesthetized with sodium pentobarbital (Nembutal) injected intravenously. The liver was perfused with the nonrecycling, hemoglobin-free system described previously (14) except that the liver was cannulated in the porta hepatitis and the hepatic vein was cut to allow the medium to pass through the liver. The surgery was completed and the isolated liver fully perfused within 3 min after the body cavity was opened. Samples of the perfusate were taken for the determination of metabolites at intervals during the perfusion. Glucose, lactate, acetoacetate, and β-hydroxybutyrate were determined by standard, enzymatic procedures (15).

**Preparation of Hepatocytes**—Hepatocytes were isolated from the livers of 48-h starved chickens according to the method of Berry and Friend (16) with the modifications introduced by Krebs et al. (17) and Sainsbury (18) and adapted for the chicken by Ohis and Harris (19). The hepatocytes were incubated at 37°C in Krebs-Henseleit bicarbonate buffer, pH 7.4, containing substrates at concentrations noted in Table I, in 25-ml Erlenmeyer flasks sealed with a serum stopper equipped with a plastic center well (Kontes). After 30 min of incubation the reaction was terminated by the injection of perchloric acid through the cap and, in some of the experiments in this study, 14CO2 was collected in 0.4 ml of hyamine in the center well (20).

**Measurement of Metabolites in Liver**—The metabolites listed in Table III were measured by enzymatic procedures following techniques detailed by Bergmeyer (15). They were isolated from perfused liver which was "freeze-stopped" at the times indicated in Table IV. The method for extraction of the metabolites from the freeze-stopped liver sample has been described in detail in a previous publication (20).
put of acetoacetate and β-hydroxybutyrate by perfused chicken liver. As the concentration of pyruvate is increased to 0.5 mM and above, the synthesis of acetoacetate is decreased, followed by a rapid fall in β-hydroxybutyrate production. At the same time, the release of lactate by the perfused liver increases with increasing pyruvate infusion. After pyruvate infusion was terminated at 90 min, the production of β-hydroxybutyrate (but not acetoacetate) from octanoate returns to normal levels. When the same experiment is repeated with lactate as the substrate (Fig. 3), the net synthesis of ketone bodies from octanoate was not affected until the concentration of lactate in the perfusate reached 2–10 mM. At this concentration rates of gluconeogenesis from lactate were very high (about 2.5 μmol of glucose synthesized/min/g of liver), but the total output of β-hydroxybutyrate was about one-third of that noted at lower (0.5 mM) lactate concentrations. Thus, the effect of these substrates on ketogenesis from octanoate is independent of possible changes in the cytosol NAD/NADH ratio caused by lactate oxidation, since both lactate and pyruvate inhibit ketone body synthesis. However, pyruvate is effective at lower concentrations, suggesting that the intracellular pyruvate concentration may determine the net rate of acetyl-CoA conversion to ketone bodies by mitochondria from chicken liver.

It is interesting to note the pattern of ketone body synthesis in these experiments. Chicken liver synthesized about twice as much β-hydroxybutyrate as compared with acetoacetate when octanoate alone was perfused (Figs. 2 and 3). The addition of either pyruvate or lactate to the perfusion medium, at relatively low concentrations (0.3–0.5 mM) caused a rapid decline in acetoacetate and a rise in β-hydroxybutyrate output. As the concentration of either lactate or pyruvate was increased further, β-hydroxybutyrate synthesis also decreased. There was, however, a marked difference in the pattern of ketone body synthesis depending upon whether dihydroxyacetone or glycerol was the gluconeogenic substrate. With dihydroxyacetone, as with lactate, there was a high rate of hepatic glucose synthesis (Fig. 4A) and β-hydroxybutyrate was the predominant ketone body formed. With glycerol, however, there was 4 times more acetoacetate formed than β-hydroxybutyrate but a relatively lower rate of gluconeogenesis (Fig. 4B).

In order to determine the interaction between pyruvate metabolism and ketogenesis from octanoate, isolated hepatocytes were incubated with [1-14C]octanoate and unlabeled pyruvate or either [1-14C]pyruvate or [2-14C]pyruvate and unlabeled octanoate. The output of 14CO2 as well as the net synthesis of glucose, lactate, acetoacetate, and β-hydroxybutyrate was determined (Table 1). This technique permits an independent assessment of the net flux of each precursor to ketone bodies and CO2 and the effect that the metabolism of one has on the other.

The oxidation of [1-14C]octanoate to 14CO2 by chicken hepatocytes occurs at a low rate which can be stimulated by the addition of unlabeled pyruvate. These hepatocytes also synthesize 8 times as much acetoacetate as β-hydroxybutyrate. The addition of pyruvate stimulates octanoate oxidation to 14CO2 by 5.4-fold while greatly decreasing net ketone body synthesis. It is interesting that pyruvate, while decreasing total ketone body formation, actually doubled β-hydroxybutyrate synthesis. The oxidation of [1-14C]pyruvate to 14CO2

*FIG. 2. Effect of increasing concentrations of pyruvate on the synthesis of glucose, ketone bodies, and lactate by perfused chicken liver.* Pyruvate, at the concentrations noted in the figure, was infused into the liver approximately 15 min after the initial infusion of 0.2 mM octanoate (Oct). The perfusion effluent was sampled at 5-min intervals and the various products determined as outlined under “Experimental Procedures.” The animals were starved for 48 h prior to use. Values are the means ± S.E. for 5 separate experiments. BOH, β-hydroxybutyrate; AcAc, acetoacetate.

*FIG. 3. Effect of increasing concentrations of lactate on the synthesis of glucose and ketone bodies by perfused chicken liver.* The experimental conditions were outlined in the legend to Fig. 2. Values are the means ± S.E. for 5 separate experiments.

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In the experiments with isolated hepatocytes, we did not determine the relative contribution of pyruvate and octanoate to ketone body synthesis when both substrates were present together, since the low level of total ketogenesis under these conditions made accurate measurements difficult. Isolated chicken liver mitochondria were used to further assess this point since these mitochondria, unlike chicken hepatocytes, synthesize only acetoacetate, with only marginal levels of β-hydroxybutyrate detectable. The radioactivity in [1-14C]acetocacetate and 14CO2, and net acetocacetate synthesis were determined when both pyruvate and octanoate were present, and a comparison made between chicken and guinea pig liver mitochondria (Table II).

As we noted with chicken hepatocytes, the rate of ketone body synthesis from octanoate by mitochondria exceeded that from pyruvate. However, acetocacetate production by chicken liver mitochondria was not significantly decreased when both octanoate and pyruvate were added together. The same pattern was noted for guinea pig liver mitochondria, except that the overall level of ketone body synthesis from pyruvate was lower.

The conversion of acetyl-CoA derived from [2-14C]pyruvate into [14C]acetocacetate by chicken liver mitochondria was not decreased by octanoate, whereas its oxidation to 14CO2 was markedly diminished. This finding is surprising since the net synthesis of acetoacetate is increased 3-fold by octanoate. In contrast, octanoate decreased the oxidation of [1-14C]pyruvate to 14CO2 by chicken liver mitochondria to about 25% of the level noted with pyruvate alone. This means that despite the reduction in acetyl-CoA formation from pyruvate, the overall conversion of the pyruvate-derived acetyl-CoA to ketone bodies (as measured by the incorporation of [2-14C]pyruvate into acetoacetate) was not decreased by octanoate. This conclusion is supported by experiments in which [1-14C]octanoate conversion to [13C]acetoacetate and 14CO2 by chicken liver mitochondria was determined when unlabelled pyruvate was present. In this case pyruvate decreased by 30% the conversion of acetyl-CoA derived from [1-14C]octanoate into acetoacetate but had no effect on total ketone body synthesis.

The pattern of interaction between pyruvate and octanoate metabolism by guinea pig liver mitochondria is very different. Octanoate decreases both the oxidation of pyruvate to CO2 via pyruvate dehydrogenase, as shown by the low total rate of 14CO2 production from [1-14C]pyruvate in the presence of this fatty acid, and also blocks the conversion of pyruvate-derived acetyl-CoA into ketone bodies (as measured by the low incorporation of [2-14C]pyruvate into acetoacetate). Thus, the sparing effect of octanoate oxidation on the utilization of pyruvate which occurs in the livers of mammalian species, such as the guinea pig, does not occur to the same extent in avian liver mitochondria.

The interaction between lactate or pyruvate utilization and the metabolism of octanoate was further studied by analyzing the intermediates of gluconeogenesis and the citric acid cycle in perfused chicken livers. When single substrates such as lactate, pyruvate, or octanoate were studied the perfusion was terminated after 50 min by "freeze-stopping." In separate experiments in which the effect of infused octanoate on pyruvate or lactate metabolism was studied, the perfused livers were freeze-stopped at 70 min. Control livers were freeze-stopped at 30 min. The ATP/ADP ratio ranged from 2-3 depending upon which substrate was perfused and the concentration of AMP was always about 10% of the ATP concentration (Table III).

When 2 mM lactate was perfused and the liver freeze-stopped, most of the intermediates on the pathway of gluconeogenesis increased (Table III). The most notable changes were in the concentrations of α-glycerophosphate and P-enol-
pyruvate. Also, the levels of malate increased almost 5-fold. The simultaneous infusion of octanoate with lactate greatly elevated the concentration of α-glycerophosphate to 8 times that noted in the absence of substrates. Citric acid cycle intermediates, malate, citrate, and α-ketoglutarate were also dramatically increased. However, octanoate actually decreased the P-enolpyruvate concentration below that noted with lactate alone. The metabolism of pyruvate by perfused chicken livers resulted in a different pattern of metabolite concentrations after freeze-stopping. The most striking effect was the 10-fold increase in P-enolpyruvate to almost 0.7 mmol/g of liver. All of the citric acid cycle intermediates were increased in concentration by pyruvate infusion, with malate reaching levels of 3 mmol/g (6 mm). The simultaneous infusion of octanoate and pyruvate had only a marginal effect on the concentration of gluconeogenic intermediates as compared to livers perfused with pyruvate alone. However, intermediates of the citric acid cycle, including citrate, malate, α-ketoglutarate, and oxaloacetate reached exceedingly high levels. For example, the concentration of oxaloacetate under these condi-
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Effect of octanoate on the concentration of various intracellular intermediates in glucose synthesis from dihydroxyacetone and glycerol by perfused chicken liver

Livers from 48-h starved chickens were perfused with Krebs-Henseleit buffer, pH 7.4, containing various substrates; then the livers were rapidly frozen using tongs cooled in liquid N (no substrate). When 2 mM dihydroxyacetone or 2 mM glycerol was infused, the livers were freeze-stopped at 50 min. With 0.2 mM octanoate the perfusion was terminated by freeze-stopping at 70 min.

Table IV

<table>
<thead>
<tr>
<th>Metabolite measured</th>
<th>Dihydroxyacetone</th>
<th>Dihydroxyacetate + octanoate</th>
<th>Glycerol</th>
<th>Glycerol + octanoate</th>
<th>pmol/g liver</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose</td>
<td>0.915 ± 0.147</td>
<td>0.382 ± 0.042</td>
<td>0.317 ± 0.084</td>
<td>0.171 ± 0.017</td>
<td></td>
</tr>
<tr>
<td>Glucose-6-P</td>
<td>0.024 ± 0.005</td>
<td>0.017 ± 0.002</td>
<td>0.011 ± 0.002</td>
<td>0.004 ± 0.002</td>
<td></td>
</tr>
<tr>
<td>Fructose-1,6-di-P</td>
<td>0.020 ± 0.005</td>
<td>0.025 ± 0.001</td>
<td>0.012 ± 0.001</td>
<td>0.009 ± 0.001</td>
<td></td>
</tr>
<tr>
<td>Dihydroxyacetone-P</td>
<td>0.048 ± 0.006</td>
<td>0.053 ± 0.007</td>
<td>0.032 ± 0.003</td>
<td>0.026 ± 0.005</td>
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</tr>
<tr>
<td>Glyceraldehyde-3-P</td>
<td>0.040 ± 0.003</td>
<td>0.025 ± 0.002</td>
<td>0.016 ± 0.003</td>
<td>0.013 ± 0.002</td>
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</tr>
<tr>
<td>α-Glycerophosphate</td>
<td>0.189 ± 0.028</td>
<td>0.572 ± 0.079</td>
<td>2.510 ± 0.443</td>
<td>5.126 ± 0.276</td>
<td></td>
</tr>
<tr>
<td>P-enolpyruvate</td>
<td>0.217 ± 0.011</td>
<td>0.087 ± 0.006</td>
<td>0.065 ± 0.005</td>
<td>0.042 ± 0.006</td>
<td></td>
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<tr>
<td>Pyruvate</td>
<td>0.059 ± 0.003</td>
<td>0.043 ± 0.003</td>
<td>0.027 ± 0.001</td>
<td>0.039 ± 0.004</td>
<td></td>
</tr>
<tr>
<td>Lactate</td>
<td>0.172 ± 0.013</td>
<td>0.056 ± 0.008</td>
<td>0.144 ± 0.029</td>
<td>0.049 ± 0.016</td>
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<tr>
<td>Oxalacetate</td>
<td>0.028 ± 0.002</td>
<td>0.023 ± 0.005</td>
<td>0.019 ± 0.004</td>
<td>0.010 ± 0.003</td>
<td></td>
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<tr>
<td>Citrate</td>
<td>1.048 ± 0.100</td>
<td>1.444 ± 0.046</td>
<td>0.038 ± 0.004</td>
<td>0.030 ± 0.010</td>
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<tr>
<td>α-Ketoglutarate</td>
<td>0.237 ± 0.017</td>
<td>0.348 ± 0.070</td>
<td>0.130 ± 0.017</td>
<td>0.022 ± 0.002</td>
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</tr>
<tr>
<td>Malate</td>
<td>0.267 ± 0.018</td>
<td>0.258 ± 0.026</td>
<td>0.083 ± 0.005</td>
<td>0.023 ± 0.006</td>
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</tr>
<tr>
<td>ATP</td>
<td>1.61 ± 0.12</td>
<td>1.99 ± 0.13</td>
<td>1.92 ± 0.16</td>
<td>2.04 ± 0.15</td>
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<tr>
<td>ADP</td>
<td>1.01 ± 0.04</td>
<td>0.82 ± 0.05</td>
<td>1.02 ± 0.04</td>
<td>0.91 ± 0.03</td>
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</tr>
<tr>
<td>AMP</td>
<td>0.50 ± 0.05</td>
<td>0.31 ± 0.05</td>
<td>0.36 ± 0.04</td>
<td>0.24 ± 0.01</td>
<td></td>
</tr>
</tbody>
</table>

The difference in the rates of gluconeogenesis from lactate as compared to pyruvate in perfused chicken liver is illustrative of the important metabolic limitations imposed by the almost totally mitochondrial location of P-enolpyruvate carboxykinase in avian liver (2-4). In the chicken, reducing equivalents generated inside the mitochondria by fatty acid oxidation cannot be transferred to the cytosol via the malate shuttle because the oxalacetate formed from the oxidation of malate by NAD-malate dehydrogenase cannot be subsequently converted to P-enolpyruvate (due to negligible levels of P-enolpyruvate carboxykinase in the cytosol of chicken liver). Since oxalacetate itself, or aspartate which might be formed from oxalacetate via cytosol aspartate aminotransferase, cannot re-enter the mitochondria at significant rates, it is not possible to transport reducing equivalents from the mitochondria to the cytosol by the conventional anion shuttles known to occur in mammalian liver. If NADH is generated directly in the cytosol, as, for example, during ethanol oxidation, the rate of pyruvate conversion to glucose is markedly stimulated. In general, lactate and dihydroxyacetone were the best substrate for glucose synthesis (5-7), whereas only marginal rates of hepatic gluconeogenesis were noted for any of an extensive list of amino acids tested by Brady et al. (21).

The regulation of hepatic ketogenesis in birds is also very different than noted in mammalian liver. Soling et al. (22) reported that, in contrast to rat liver, starvation did not increase hepatic ketogenesis in pigeons. They suggested that the hepatic disposal of long chain free fatty acids by pigeon liver is regulated in such a way that the hepatic supply of ketone bodies can be kept constant over a wide range of nutritional states. The studies reported in the present paper suggest an explanation for this observation. The overall rate of ketogenesis by perfused chicken liver is very sensitive to the concentration of pyruvate (Fig. 2). At levels of pyruvate between 0.5 and 1 mM the synthesis of ketone bodies from octanoate is greatly diminished. Furthermore, lactate also can inhibit ketogenesis, but only when its concentration in the perfusate exceeds 2 mM (Fig. 3). The reason for this dramatic effect may be due to the stimulatory action of pyruvate on octanoate oxidation to CO2 and water noted with isolated hepatocytes (Table I). Our data with freeze-stopped livers which were perfused with octanoate and pyruvate indicate a build-up of citric acid cycle intermediates, especially citrate, malate, ketoglutarate, and oxalacetate to extremely high levels. This is probably due to the inability of the chicken liver mitochondria to transport reducing equivalents out of the mitochondria to support gluconeogenesis from oxidized substrates such as pyruvate. It seems likely that some portion of...
the acetyl-CoA formed from fatty acid oxidation is diverted into the citric acid cycle when pyruvate is being simultaneously metabolized. This would decrease the net flux of acetyl-CoA into the citric acid cycle when pyruvate is being simultaneously oxidized. The acetyl-CoA formed from fatty acid oxidation is diverted into ketone body synthesis which is observed when both pyruvate and octanoate are perfused together. The same pattern of response is not observed with either rat or guinea pig livers, indicating a major species difference in the regulation of hepatic ketogenesis.

A major, unsolved problem which complicates our understanding of the regulation of ketosis in the chicken is the negligible level of β-hydroxybutyrate dehydrogenase activity in chicken liver mitochondria (7, 23). In our studies (Table I) we have measured rates of ketone body formation, from octanoate, of about 1.8 μmol/min/g wet weight of hepatocytes, 88% of which was β-hydroxybutyrate. However, isolated chicken liver mitochondria synthesize only acetocetate from octanoate (Table II) or palmitoyl carnitine (data not shown). Despite a number of attempts to measure β-hydroxybutyrate dehydrogenase activity in both mitochondria and cytosol fractions from chicken liver, we have been unable to detect significant activity of this enzyme. This may be due to inactivation during enzyme solubilization or to our failure to add a required, but, hitherto unknown, cofactor.

The ratio of acetocetate and β-hydroxybutyrate synthesis by perfused chicken liver varies with the substrate perfused. For example, with octanoate alone, both acetocetate and β-hydroxybutyrate are released by the liver (Figs. 2 and 3) and the addition of pyruvate at low concentration initially stimulates β-hydroxybutyrate synthesis while inhibiting acetocetate formation. Then, at pyruvate concentrations of 0.5 mM the synthesis of both ketone bodies is markedly depressed. The measurement of metabolites presented in Table III shows that when both pyruvate and octanoate are perfused simultaneously there is an accumulation of citric acid cycle intermediates, particularly malate, which can reach a concentration of 20 mM, suggesting that the initial response of the mitochondria after pyruvate perfusion involves a disposal of reducing equivalents by the reduction of acetocetate to β-hydroxybutyrate. The same pattern was observed with lactate perfusion (Fig. 3).

The pattern of ketone body synthesis with dihydroxyacetone or glycerol plus octanoate provides a potential insight into the regulation of ketogenesis. The net synthesis of glucose from dihydroxyacetone is relatively high, and compares favorably with rates of gluconeogenesis observed with lactate. Octanoate addition stimulates the synthesis of β-hydroxybutyrate with only a marginal rise in the levels of acetocetate output by the liver (Fig. 4). The measurement of metabolites in the liver during the combined perfusion of dihydroxyacetone and octanoate does not indicate an accumulation of citric acid cycle anions suggesting a balance in net re-oxidation of mitochondrial NADH in part due to a net synthesis and release of β-hydroxybutyrate at the rate of 1.2 μmol/min/g of liver. The perfusion of glycerol with octanoate, on the other hand, results in the predominant synthesis of acetocetate. This may be due in part to the low overall rate of glycerol utilization by chicken liver. Our freeze-stop experiments presented in Table IV show that glycerol is converted to glycero-phosphate in the chicken liver, reaching a concentration of more than 10 mM when both glycerol and octanoate are perfused together. This probably is due to a limitation in the oxidation of cytosol NADH generated when β-glycerophosphate is oxidized to dihydroxyacetone phosphate. Under these conditions the synthesis of acetocetate by chicken liver greatly exceeds the formation of β-hydroxybutyrate. Thus, the only conditions we have noted in which the ratio of acetocetate formation by perfused chicken livers exceeds the synthesis of β-hydroxybutyrate is when octanoate is metabolized alone, or in combination with glycerol. In the latter conditions the uptake of glycerol is markedly limited by the inability of the liver to oxidize α-glycerophosphate, which subsequently accumulates to high concentrations. Whenever there is a net flux through the citric acid cycle stimulated by the perfusion of substrates readily converted to pyruvate, or by pyruvate itself at concentrations below 0.5 mM, β-hydroxybutyrate is formed. Since β-hydroxybutyrate is normally released from the liver in vivo for further oxidation by peripheral tissues such as muscle and brain, this means that β-hydroxybutyrate synthesis and release may be a major route for transferring reducing equivalents from the liver to extrahepatic tissues. It is obvious that the regulation of ketogenesis in chicken liver is markedly different from that observed in the well-studied mammalian species such as the rat, guinea pig (24), and human.

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Mitochondrial phosphoenolpyruvate carboxykinase (GTP) and the regulation of gluconeogenesis and ketogenesis in avian liver.
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