Regulation of Oxalacetate Metabolism in Liver Mitochondria

EVIDENCE FOR NICOTINAMIDE ADENINE DINUCLEOTIDE-MALATE DEHYDROGENASE EQUILIBRIUM AND THE ROLE OF PHOSPHOENOLPYRUVATE CARBOXYKINASE IN THE CONTROL OF OXALACETATE METABOLISM IN INTACT GUINEA PIG AND RAT LIVER MITOCHONDRIA*

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SUMMARY

The metabolism of malate was investigated in liver mitochondria from both the rat and the guinea pig. The oxidation of this intermediate was found to proceed in an inhibited and nonlinear manner in guinea pig liver mitochondria and to a lesser extent this situation held also for rat liver mitochondria. This inhibition was reversed by glutamate and partially by pyruvate addition. Analyses of the mitochondrial suspensions throughout an ADP-induced respiratory cycle showed increasing levels of oxalacetate during the transition from State 4 to State 3, with further increases occurring throughout State 3. Inhibition of malate oxidation was associated with markedly elevated levels of oxalacetate. In guinea pig liver mitochondria with malate as substrate, a large amplitude oxidation of the respiratory chain-linked pyridine nucleotides was observed during State 3 which persisted beyond the apparent transition into State 4. A significant release of protons to the medium was noted in State 4, whereas in State 3, lower H⁺ to ADP and ADP to oxygen ratios were obtained with guinea pig as compared to rat liver mitochondria. Factors increasing the rate of oxalacetate removal in mitochondria were noted to also increase the rate of malate oxidation and the formation of reduced pyridine nucleotides during the transition from State 3 to State 4. These same factors also decreased the rate of proton release in State 4, and increased the ratios of H⁺ to ADP and ADP to oxygen.

P-enolpyruvate carboxykinase was found to function in a reversible manner in guinea pig liver mitochondria. Significant amounts of intramitochondrial oxalacetate could be formed from added P-enolpyruvate, provided that the ratio of ATP:ADP was low. Using this technique to vary the level of oxalacetate within the matrix space, marked inhibition of malate oxidation was produced by increasing the concentration of oxalacetate. In addition, the mechanisms of oxalacetate removal appear different in liver mitochondria from the guinea pig as compared to the rat. The flow of carbon from oxalacetate to P-enolpyruvate limits, in part, the State 3 rate of respiration from malate in guinea pig liver mitochondria. Such a limitation arising from P-enolpyruvate synthesis is observed despite the markedly elevated levels of oxalacetate which thereby result. Other factors such as the availability of ATP to form GTP by transphosphorylation also appear to control the rate and direction of the P-enolpyruvate carboxykinase reaction. Decreased rates of P-enolpyruvate formation inhibit the rate of malate oxidation in either State 3 or State 4. The metabolic consequences of mitochondrial P-enolpyruvate formation result in significantly altered kinetic parameters of malate metabolism in mitochondria from these two species.

It is concluded that NAD+m-alate dehydrogenase functions in an equilibrium manner in the intact liver mitochondria of both rat and guinea pig, and that the intramitochondrial pools of malate, oxalacetate, and the nicotinamide coenzymes freely interact in such a system. Alterations of any one factor produce direct and reciprocal changes in the other intermediates. Additionally, P-enolpyruvate carboxykinase although reversible, appears to function primarily as an oxalacetate-removing system. In guinea pig liver mitochondria, the presence of this enzyme results in altered control mechanisms of oxalacetate metabolism and in marked differences in the regulation of the citric acid cycle. These observations have important implications for the mechanisms of hepatic gluconeogenesis in both the rat and the guinea pig.

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A number of recent observations have suggested that the detailed mechanisms and controlling aspects of hepatic gluconeogenesis may vary in animal species. In the rat and related species in which primarily a cytosolic activity of hepatic P-enolpyruvate carboxykinase is found, glucagon infusion (1) or fatty acid addition (2, 3) in the perfused liver increases the rate of glucose synthesis from lactate. Studies in those species, such as the guinea pig, in which a significant mitochondrial activity of hepatic P-enolpyruvate carboxykinase is found, have shown gluconeogenesis to be insensitive to glucagon infusion (4). Furthermore, fatty acid infusion inhibits gluconeogenesis (4, 5) from lactate rather than enhancing it. Analysis of the steady state levels of various intermediates in the freeze-clamped livers of fed and fasted guinea pigs and rabbits has shown a marked shift...
toward oxidation of the intramitochondrial nicotinamide coenzymes during fasting (6, 7). Both species have approximately a 4-fold increase in the ratio of NAD⁺:NADH as calculated from either the β-hydroxybutyrate dehydrogenase or the l-glutamate dehydrogenase equilibria. Again, these data contrast with prior observations in rat livers, in which a shift toward reduction of the intramitochondrial oxidation-reduction potential occurs upon fasting (8). Since fasting is associated with increased rates of gluconeogenesis in vivo, the differing shifts of the intramitochondrial oxidation-reduction potential may, in fact, reflect differing control mechanisms of gluconeogenesis. It is not entirely clear whether these differences in gluconeogenesis result from the species-related variability in the intracellular distribution of P-enolpyruvate carboxykinase. Nevertheless, some alterations in mitochondrial metabolism must be expected if the intramitochondial form of the enzyme is to function for gluconeogenesis in a manner distinct from the cytosolic enzyme. In this study, the intramitochondrial control and metabolism of oxalacetate have been investigated in both guinea pig and rat liver mitochondria. The resulting alterations between species have been related to the intramitochondrial activity of P-enolpyruvate carboxykinase, and the significance of these alterations for various mechanisms of gluconeogenesis are discussed.

MATERIALS AND METHODS

**Materials**

Pyruvate kinase (EC 2.7.1.40), NAD⁺-malate dehydrogenase (EC 1.1.1.37), hexokinase (EC 2.7.1.1), glucose 6-phosphate dehydrogenase (EC 1.1.1.49), NAD⁺, and potassium P-enolpyruvate were purchased from Boehringer Mannheim. ATP, ADP, NADH, and lactate dehydrogenase (EC 1.1.1.27) were obtained from P-L Biochemicals. Malate, succinate, pyruvate, and l-glutamic acid were purchased from Calbiochem. Carbonyl cyanide m-trifluoromethoxyphenylhydrazone was a generous gift of Dr. P. Layter (Du Pont, Wilmington, Del.).

**Methods**

Isolation of Mitochondria—Using the procedure of Schneider and Hogeboom (9) as described previously (10), mitochondria were isolated from male albino guinea pigs of the Hartley strain weighing between 300 to 500 g. The homogenates were prepared in 0.225 M mannitol, 0.075 M sucrose, 0.001 M triethanolamine hydrochloride, pH 7.5, and 0.0001 M EDTA. The mitochondria were washed either once with small amounts of medium or twice with 250 ml of the mannitol-sucrose solution, omitting EDTA where indicated, and were resuspended in 0.25 M sucrose solution, omitting EDTA where indicated, and were resuspended in 0.25 M sucrose for all studies. These large wash volumes were used in order to deplete the mitochondria of most endogenous substrates. Mitochondrial nitrogen was determined by micro-Kjeldahl digestion and subsequent nesslerization (11), with corrections applied for kinases activity was detected in any mitochondrial preparation, containing approximately 2.0 mg per ml of mitochondrial protein together with 1000 μmole of sucrose, 20 μmole of potassium phosphate, and 20 μmole of MgCl₂ in a final volume of 4.0 ml. Other additions to this media included 10 to 40 μmole of the appropriate carbon substrate and variable amounts of potassium P-enolpyruvate and potassium bicarbonate where indicated. The final pH of the media was 7.5. The osmolality of the medium was 297 mosm as determined with a Fiske osmometer. Oligomycin addition, where indicated, was titrated for each mitochondrial preparation and the means are reported for the appropriate experiments. For analytical purposes, samples of the mitochondrial incubation were removed rapidly from the oxygen cuvette with an Eppendorf pipette at the points indicated and transferred directly into ice-cold 10% perchloric acid. The resulting precipitate was removed by centrifugation at 20,000 X g and supernatants quantitatively transferred to tubes containing preweighed amounts of KClO₃ such that the resultant pH of the mixture was 6.0. All extracts were maintained at 0° throughout this procedure. The total time of acidification for any single mitochondrial extract was less than 15 min. Analyses for intermediates were completed within 2 hours for all extracts.

**Assays**—Pyruvate and P-enolpyruvate were determined by modifications of the assay of Czok and Eckert (12). ATP (13) and ADP (14) were determined enzymatically. Assays for oxalacetate in mitochondrial extracts with malate dehydrogenase and NADH were adapted for use on a dual wave length spectrophotometer. A glass slide having a net ΔA of 0.020 at 340 to 380 nm and the instrument was calibrated daily with dilutions of NADH previously standardized on a Gilford model 2400 spectrophotometer. A glass slide having a net ΔA of 0.020 at 340 to 380 nm was used as an external standard. Each gain setting was adjusted and calibrated between each determination. The maximum useful sensitivity of this assay procedure was 15 pmoles of oxalacetate per ml of mitochondrial extract. Internal standards of oxalacetate or pyruvate were added to the samples for control purposes. The over-all signal to noise ratio was not less than 4:1 at maximum sensitivity. This method of estimation of oxalacetate was chosen not only for the high degree of sensitivity but also to avoid the nonspecific and unpredictable fluorescence enhancement or quenching of NADH, seen fluorometrically after the addition of NAD⁺-malate dehydrogenase (16).

Oxygen consumption was followed in a thermostated cuvette with a Clark-type oxygen electrode (YSI Instruments). Concurrent pH traces were obtained with a Radiometer combination glass electrode mounted in the same cuvette. For studies of pyridine nucleotides-linked reactions, ATPases, and light-scattering changes, a modified cuvette based on the model described by Pressman (17) was adapted to a Chance dual wave length spectrophotometer (18). Simultaneous measurements of pyridine nucleotide fluorescence were obtained with excitation at 366 nm and frontal detection of the 470-nm fluorescence at a 90° angle to the light-scattering channel (17). Light-scattering changes at 575 nm were followed on a different channel with single beam detection with dual wave length optics (19). Changes in pH and in oxygen consumption were measured as described above. These measurements were all performed simultaneously in a thermostated cuvette equipped with a stirring motor at a final volume of 9.0 ml.

**RESULTS**

Since malate is the direct precursor of oxalacetate in the citric acid cycle, the kinetic parameters of malate oxidation in both guinea pig and rat liver mitochondria have been studied to elucidate controls on the mitochondrial disposition of oxalacetate. In addition to measurements of oxygen consumption, pyridine nucleotide fluorescence of mitochondrial suspensions has been used to evaluate rapidly the availability to the respiratory chain of reducing equivalents produced by the citrate acid cycle de-
hydrogenases. Changes in pH of the incubation media were routinely measured as an independent indicator of the rate of oxidative phosphorylation. A comparison of the respiratory cycles induced by ADP addition in mitochondria from both species oxidizing malate as substrate revealed two striking points of contrast. First, the time required for completion of the cycle in guinea pig liver mitochondria (Fig. 1A) was 3-fold longer than that required by rat liver mitochondria (Fig. 1B). This was evidenced by the broadened peak in pyridine nucleotide oxidation, and by the decreased and prolonged rate of proton uptake indicating a slower rate of oxidative phosphorylation per mg of protein with the guinea pig. The second and most important point of difference is that oxygen consumption with liver mitochondria from the guinea pig was nonlinear and markedly inhibited showing a poorly defined transition from State 3 (respiration maximally stimulated by ADP) into State 4 (respiration limited by ADP). At the end of State 3 respiration, reduction of cytochrome a showed approximately the same amount of pyridine nucleotide oxidation maximally stimulated by ADP) into State 4 (respiration limited by ADP). However, at the decreased and prolonged rate of proton uptake indicating a slower rate of oxidative phosphorylation per mg of protein with the guinea pig. The second and most important point of difference is that oxygen consumption with liver mitochondria from the guinea pig was nonlinear and markedly inhibited showing a poorly defined transition from State 3 (respiration maximally stimulated by ADP) into State 4 (respiration limited by ADP). At the end of State 3 respiration, reduction of cytochrome a showed approximately the same amount of pyridine nucleotide oxidation maximally stimulated by ADP) into State 4 (respiration limited by ADP). However, the observation that this species contains only one-half the amount of respiratory cytochromes as does the rat (20). As a result, a decreased maximal rate of respiration in State 3 might then be expected. Spectral determinations of pyridine nucleotide and cytochrome content (21) in our own preparations of mitochondrial suspension showed approximately the same amount of pyridine nucleotides in both species, and confirmed that the cytochrome content in the guinea pig may be half that of the rat. However, a limitation on the maximal rates of respiration in guinea pig mitochondria due to this decreased content of cytochromes does not appear likely since the addition of 5 mM glutamate significantly shortened the duration of the subsequent respiratory cycle and increased the rates of both oxygen consumption and proton uptake in State 3. These determinations were linear in nature and oxygen consumption showed a well defined transition from State 3 to State 4. The effects of glutamate addition were much more pronounced in guinea pig than in rat liver mitochondria which evidenced only minimal inhibition of malate oxidation. Endogenous glutamate was not detectable in either mitochondrial preparation. In fact, the over-all kinetic data of malate plus glutamate oxidation in State 3 appear quite similar in both species.

Other mitochondrial substrates were tested for their effects on the kinetics of malate oxidation. In Fig. 2, the results of pyruvate addition to guinea pig (A) and rat (B) liver mitochondria are shown. The initial rates of oxygen consumption and proton uptake in State 3 were increased somewhat by pyruvate addition, although its effect on these processes was far less than that of glutamate. Nevertheless, a well defined transition from State 3 to State 4 was established in both types of mitochondria by either pyruvate or glutamate. The differences in effectiveness between glutamate and pyruvate may be related to the extent of pyridine nucleotide reduction produced by their addition in State 4. With malate alone as substrate, State 4 levels of pyridine nucleotide oxidation were consistently greater after an ADP cycle (State 4) than before such a respiratory cycle (State 4). This was true for mitochondria of both species. Glutamate addition (Fig. 1) resulted in a prompt reduction of these nucleotides to an extent greater even than that observed initially (State 4) with malate alone. Pyruvate addition under these same conditions (Fig. 2) produced only a slight reduction. Taken together, the foregoing data suggest that the inhibition of respiration from malate alone as substrate may result from a decreased availability of reducing equivalents to the respiratory chain.

In view of the reversibility of these inhibitions by glutamate, and partially by pyruvate addition, it is possible to hypothesize that malate oxidation leads to an intramitochondrial accumulation of oxalacetate which becomes inhibitory toward the further oxidation of malate. This then may reduce the rate of input of reducing equivalents to the electron transport chain, thereby decreasing the rate of respiration. To test this, mitochondrial sus-

FIG. 1. Pyridine nucleotide fluorescence, proton movements, and oxygen consumption by mitochondrial suspensions from guinea pig (A) and rat liver (B) oxidizing malate. Mitochondria (2.0 mg per ml) were incubated in 0.25 M sucrose containing 10 mM malate, 5 mM K2HPO4, 5 mM KCl, 1 mM MgCl2, and 2 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid, pH 7.5. The additions of ADP (125 mM), glutamate (glut.) (5 mM), and carbonyl cyanide p-trifluoromethoxyphenylhydrazone (FCCP) (0.2 mM) are indicated by the appropriate arrows. Calibrations of pyridine nucleotide oxidation-reduction alterations are expressed as micromoles per liter. Changes in pH of the mitochondrial suspensions are calibrated as indicated, with a scale of increasing proton release (acidification of the medium) toward the top of the figure. Numerical values appearing in the figures are the calculated rates per s for the appropriate kinetic measurements at the points indicated. Other determinations as indicated under "Methods."

FIG. 2. Pyridine nucleotide fluorescence, proton movements, and oxygen consumption in mitochondrial suspensions from guinea pig liver (A) and rat liver (B) oxidizing malate. All other details as in Fig. 1 and "Methods" except for pyruvate (pyr.) addition (5 mM).
samples of the mitochondrial suspensions were obtained for experiments, while oxygen consumption is given in micromolar nitrogen, and are the means ± standard error for at least three nearly equal (Samples B and G), although these concentrations represent a 3-fold increase over that noted during the preceding State 4 level (Samples A and F). Later, in the inhibited phase of State 3 respiration from malate and throughout the apparent transition into State 4 (Samples C, D, and E) oxalacetate was greatly elevated compared to State 3 levels in the presence of pyruvate (Samples G and H). The elevated concentration in State 4 (Sample E) was not decreased substantially until pyruvate was added to the medium and even then at a slow rate. No comparably large elevations were observed subsequently in State 4 samples with malate and pyruvate as substrates. The addition of glutamate to mitochondrial suspensions oxidizing malate in State 4 reduced the level of oxalacetate below the minimal concentration which may be detected by this assay procedure (<3 μM), although in State 3, maximal increases in levels of oxalacetate to 5.9 μM were noted. Similar data were obtained with rat liver mitochondria except that the accumulation of oxalacetate in late State 3 respiration from malate alone as substrate was not observed (Samples D and E), as noted above in the experiments with guinea pig mitochondria. Otherwise, oxalacetate levels were essentially identical. Trace II shows an initial respiratory cycle with malate plus pyruvate as substrate. No inhibition of State 3 respiration was observed indicating that the inhibition of malate oxidation does not depend on which respiratory cycle was studied.

The foregoing data suggest that the inhibition of State 3 respiration in mitochondria from guinea pig liver, relative to that noted with mitochondria from rat liver, may not result from any intrinsic, quantitative differences in electron transport or in the mechanism of oxidative phosphorylation. Rather, this inhibition seems more closely related to an intramitochondrial accumulation of oxalacetate which is greater in mitochondria from guinea pig liver than rat liver. It follows then, that the rate of forward flux through NAD+-malate dehydrogenase and hence, the availability of reducing equivalents to the electron transport chain, can become dependent upon the rate of oxalacetate removal by the enzyme systems of the citric acid cycle. Since guinea pig liver mitochondria contain a nearly 50-fold higher activity of P-enolpyruvate carboxykinase than rat liver mitochondria (5.1 versus 0.1 units per g) (5), and since this enzyme may constitute an important route for the metabolism of intramitochondrially generated oxalacetate (6), the effect of inhibition of this enzyme was studied in the various mitochondrial preparations. Inhibition was produced by increasing CO₂ in the medium by the inclusion of bicarbonate which establishes a significant availability of reducing equivalents to the electron transport chain. Since guinea pig liver mitochondria contain an approximately 75-fold higher activity of P-enolpyruvate carboxykinase than rat liver mitochondria, the rate of forward flux through NAD+-malate dehydrogenase and hence, the availability of reducing equivalents to the electron transport chain, can become dependent upon the rate of oxalacetate removal by the enzyme systems of the citric acid cycle. Since guinea pig liver mitochondria contain a nearly 50-fold higher activity of P-enolpyruvate carboxykinase than rat liver mitochondria (5.1 versus 0.1 units per g) (5), and since this enzyme may constitute an important route for the metabolism of intramitochondrially generated oxalacetate (6), the effect of inhibition of this enzyme was studied in the various mitochondrial preparations. Inhibition was produced by increasing CO₂ in the medium by the inclusion of bicarbonate which establishes a significant availability of reducing equivalents to the electron transport chain. Since guinea pig liver mitochondria contain a nearly 50-fold higher activity of P-enolpyruvate carboxykinase than rat liver mitochondria (5.1 versus 0.1 units per g) (5), and since this enzyme may constitute an important route for the metabolism of intramitochondrially generated oxalacetate (6), the effect of inhibition of this enzyme was studied in the various mitochondrial preparations. Inhibition was produced by increasing CO₂ in the medium by the inclusion of bicarbonate which establishes a significant availability of reducing equivalents to the electron transport chain. Since guinea pig liver mitochondria contain a nearly 50-fold higher activity of P-enolpyruvate carboxykinase than rat liver mitochondria (5.1 versus 0.1 units per g) (5), and since this enzyme may constitute an important route for the metabolism of intramitochondrially generated oxalacetate (6), the effect of inhibition of this enzyme was studied in the various mitochondrial preparations. Inhibition was produced by increasing CO₂ in the medium by the inclusion of bicarbonate which establishes a significant availability of reducing equivalents to the electron transport chain. Since guinea pig liver mitochondria contain a nearly 50-fold higher activity of P-enolpyruvate carboxykinase than rat liver mitochondria (5.1 versus 0.1 units per g) (5), and since this enzyme may constitute an important route for the metabolism of intramitochondrially generated oxalacetate (6), the effect of inhibition of this enzyme was studied in the various mitochondrial preparations. Inhibition was produced by increasing CO₂ in the medium by the inclusion of bicarbonate which establishes a significant availability of reducing equivalents to the electron transport chain. Since guinea pig liver mitochondria contain a nearly 50-fold higher activity of P-enolpyruvate carboxykinase than rat liver mitochondria (5.1 versus 0.1 units per g) (5), and since this enzyme may constitute an important route for the metabolism of intramitochondrially generated oxalacetate (6), the effect of inhibition of this enzyme was studied in the various mitochondrial preparations. Inhibition was produced by increasing CO₂ in the medium by the inclusion of bicarbonate which establishes a significant availability of reducing equivalents to the electron transport chain.

**Fig. 3.** Oxygen consumption in guinea pig liver mitochondrial suspensions oxidizing malate. All other details as in Fig. 1. Rapid sampling as described under "Methods" was obtained where indicated (*) by series A through I, as noted in Table I. PYR, pyruvate.

**Table I**

**Level of intramitochondrial oxalacetate during mitochondrial respiration with malate**

Guinea pig liver mitochondrial suspensions oxidizing malate (10 mM) in the oxygen cuvette were sampled at the points indicated in Fig. 3. Pyruvate was added to a final concentration of 5 mM where indicated. ADP addition was 250 μM. Samples were rapidly removed, acidified, and oxalacetate determined on the resulting neutralized extracts (see "Methods"). Separate samples of the mitochondrial suspensions were obtained for protein and nitrogen determination. The mean mitochondrial matrix volume was calculated from the data of Pfaff et al. (44). Oxalacetate data are given as nanomoles per mg of mitochondrial nitrogen, and are the means ± standard error for at least three experiments, while oxygen consumption is given in micromolar per s per mg of protein.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Respiratory state</th>
<th>Oxygen consumption</th>
<th>Oxalacetate</th>
</tr>
</thead>
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<tr>
<td></td>
<td>μM/s/mg protein</td>
<td>mole/mg nitrogen</td>
<td>μM</td>
</tr>
<tr>
<td>A</td>
<td>41</td>
<td>0.049 ± 0.007</td>
<td>0.086 ± 0.012</td>
</tr>
<tr>
<td>B</td>
<td>3 initial</td>
<td>0.702 ± 0.059</td>
<td>0.214 ± 0.047</td>
</tr>
<tr>
<td>C</td>
<td>3 middle</td>
<td>0.342 ± 0.047</td>
<td>0.783 ± 0.089</td>
</tr>
<tr>
<td>D</td>
<td>3 inhibited</td>
<td>0.317 ± 0.021</td>
<td>0.748 ± 0.061</td>
</tr>
<tr>
<td>E</td>
<td>42</td>
<td>0.291 ± 0.022</td>
<td>0.344 ± 0.026</td>
</tr>
<tr>
<td>F</td>
<td>42 (+ pyruvate)</td>
<td>0.294 ± 0.018</td>
<td>0.672 ± 0.009</td>
</tr>
<tr>
<td>G</td>
<td>3 initial (+ pyruvate)</td>
<td>0.816 ± 0.074</td>
<td>0.206 ± 0.029</td>
</tr>
<tr>
<td>H</td>
<td>3 middle (+ pyruvate)</td>
<td>0.810 ± 0.082</td>
<td>0.227 ± 0.035</td>
</tr>
<tr>
<td>I</td>
<td>43 (+ pyruvate)</td>
<td>0.292 ± 0.011</td>
<td>0.066 ± 0.010</td>
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**Fig. 4.** Effect of bicarbonate addition (10 mM) on pyridine nucleotide fluorescence, proton movements, and oxygen consumption in mitochondrial suspensions from guinea pig liver (A) and rat liver (B). Other details as in Fig. 1. Glutamate (glut.) addition was 5 mM. FCCP, carbonyl cyanide p-trifluoromethoxyphenylhydrazone.
P-enolpyruvate formation, as determined by serial analyses of the mitochondrial suspensions, was reduced from 7.26 to 4.09 nmoles per mg of protein per min. In the pyridine nucleotide trace, a slow pronounced oxidation was noted at the beginning of State 3 (cf. Fig. 1), with essentially no reduction during the transition from State 3 to 4. Only slight effects of bicarbonate addition were noted on rat liver mitochondria oxidizing malate. Glutamate addition produced a large reduction of the pyridine nucleotides in both species similar to that noted previously in Fig. 1. Interestingly, the State 3 rate of respiration in guinea pig mitochondria from malate plus glutamate in the presence of bicarbonate was significantly decreased when compared to rates obtained in the absence of bicarbonate, and was associated with a decreased amplitude of the oxidation-reduction cycle of the pyridine nucleotides. No significant changes were produced by bicarbonate in rat liver mitochondria using malate and glutamate as substrates, suggesting that the differences between species in their sensitivity to bicarbonate may well be related to the effects of a reduction in the rate of oxaloacetate removal via P-enolpyruvate carboxykinase in guinea pig liver mitochondria.

In contrast to the reversal by glutamate of most of the effects of bicarbonate addition, pyruvate was significantly less effective, producing primarily an increased rate of phosphorylation, as measured by proton uptake during State 3 (Fig. 3). Little or no increase in oxygen consumption, or in the rate of pyridine nucleotide reduction was observed. It is significant to note that in State 3, a persistent acidification of the medium was found with guinea pig liver mitochondria. Glutamate addition blocked this release of protons whereas pyruvate addition was less effective, whether or not bicarbonate was included in the medium. Malate plus pyruvate oxidation by guinea pig liver mitochondria in the presence of bicarbonate, appears to have resulted in a respiratory state in which phosphorylation proceeded without any apparent cycling of the pyridine nucleotides or large changes in oxygen consumption. This may reflect a steady state balance between a limiting supply of reducing equivalents to the respiratory chain, in the presence of saturating quantities of exogenous ADP. Thus, flow in the electron transport chain becomes highly limited owing to this limitation on the supply of NADH rather than the capability of added ADP to remove it. In this instance, oxaloacetate accumulation seems to be the limiting factor for the oxidation of malate. In addition, the nucleoside diphosphokinase reaction with the GDP formed during P-enolpyruvate synthesis may provide sufficient endogenous ADP to maintain a significant activation of the respiratory chain, particularly during the transition from State 3 to State 4. This effect is far more prominent at this point in the respiratory cycle since the rate of oxaloacetate and hence P-enolpyruvate formation increases several-fold in State 3. These results suggest then that P-enolpyruvate synthesis is the principal mechanism for oxaloacetate removal and can constitute a significant limitation on both malate oxidation and the over-all rate of flow in the citric acid cycle.

The relationship between oxaloacetate removal via P-enolpyruvate carboxykinase and the kinetics of malate oxidation may be further investigated by studying the effects of direct P-enolpyruvate addition to guinea pig liver mitochondrial suspensions oxidizing malate, in the presence and absence of pyruvate (Table II). Since P-enolpyruvate carboxykinase is a reversible enzyme, increased amounts of P-enolpyruvate in the

<table>
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<tr>
<th>Substrate</th>
<th>P-enolpyruvate addition</th>
<th>State 4</th>
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<tr>
<td></td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Malate</td>
<td>O$_2$ consumption, initial rate</td>
<td>0.059 ± 0.007</td>
<td>0.031 ± 0.003</td>
<td>0.792 ± 0.059</td>
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<td></td>
<td>P:O ratio</td>
<td>2.22 ± 0.04</td>
<td>5.95</td>
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<td></td>
<td>Respiratory control ratio</td>
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<tr>
<td>Malate + pyruvate</td>
<td>O$_2$ consumption, initial rate</td>
<td>0.184 ± 0.005</td>
<td>0.130 ± 0.038</td>
<td>0.966 ± 0.082</td>
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<tr>
<td></td>
<td>P:O ratio</td>
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<tr>
<td></td>
<td>Respiratory control ratio</td>
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**Fig. 5.** Effect of bicarbonate (10 µM) on pyridine nucleotide fluorescence, proton movements, and oxygen consumption in guinea pig liver mitochondrial suspensions oxidizing malate. Other details as in Fig. 1 and "Methods." Pyruvate (pyr.) addition was 5 mM, glut., glutamate.
The presence of bicarbonate should not only inhibit oxalacetate removal but may also form substantial amounts of this intermediate in the matrix space. Five millimolar P-enolpyruvate had a slight inhibitory effect on State 4 respiration by guinea pig liver mitochondria from either malate alone or in combination with pyruvate, whereas a marked inhibition of State 3 respiration was produced with either substrate. With malate alone, P:O ratios in guinea pig liver mitochondria were considerably lower than those obtained with rat liver mitochondria (2.22 versus 2.83). Pyruvate addition increased this ratio, even when P-enolpyruvate was present. P:O ratios from malate in the presence of P-enolpyruvate could not be easily evaluated, since no transition from State 3 to State 4 could be clearly discerned. This result is further explored in Fig. 6. The addition of P-enolpyruvate to guinea pig liver mitochondrial suspensions oxidizing malate slightly inhibited oxygen consumption in State 4. The rate of respiration increased about 4-fold following ADP addition but returned very rapidly to an inhibited rate which was only 50% greater than that noted during the prior State 4. No transition from State 3 into State 4 was ever observed even in the presence of pyruvate, and this form of inhibited State 3 respiration tended to persist until oxygen in the medium was exhausted. While in State 3, the addition of pyruvate increased the respiratory rate 60%. After glutamate addition, the rate of respiration increased 80%. P-enolpyruvate addition induced a rapid oxidation step of the pyridine nucleotides in which the amount of nucleotide oxidized (0.45 μmole per mg of protein) was approximately equivalent to the amount reduced when malate was added to the mitochondria in State 2 (not shown). The transition into State 3 produced a rapid oxidation of the pyridine nucleotides, but the respiratory cycle proceeded quite slowly. Pyruvate was added before all the ADP was consumed and it can be seen that the nicotinamide co-enzymes remained oxidized for approximately 50 s before the transition into State 4 occurred. At this point, the relative ratio of NAD:NADH remained 31%, oxidized, a value similar to the levels obtained when malate alone was studied (Fig. 1), but much lower than the 70% levels of oxidation when bicarbonate was added (Fig. 4). The addition of glutamate further reduced the pyridine nucleotides to an oxidation level of only 23%. In general, the inhibition of respiration produced by bicarbonate and P-enolpyruvate were quantitatively similar, although the relief of respiration and pyridine nucleotide reduction by pyruvate addition was more rapid with P-enolpyruvate. No effect of added P-enolpyruvate was noted when rat liver mitochondria were tested, indicating an absolute requirement for high levels P-enolpyruvate carboxykinase for these observations. Based on these results, it seems likely that, in guinea pig liver mitochondria, the presence of P-enolpyruvate carboxykinase can determine, in part, the oxidation-reduction potential of the pyridine nucleotides regardless of respiratory state.

The apparent relationships between P-enolpyruvate metabolism, the levels of intramitochondrial oxalacetate, and the adenine nucleotide pools, have been further studied in guinea pig liver mitochondrial suspensions oxidizing malate and treated with oligomycin. Since the formation of oxalacetate from P-enolpyruvate produces a stoichiometric amount of GTP, the regeneration of GDP by nucleoside diphosphokinase can be made the rate-limiting step for oxalacetate synthesis. From these considerations, it follows that in non-phosphorylating mitochondria, variations in the relative proportion of ATP to ADP may produce marked changes in the rate of oxalacetate formation from P-enolpyruvate. This was observed in Fig. 7 and Table III. Oligomycin addition characteristically increased the State 4 rate of respiration from malate in guinea pig liver mitochondria but decreased it in rat liver mitochondria. The addition of P-enolpyruvate to guinea pig liver mitochondria treated with oligomycin, malate, and ADP resulted in increased levels of Intramitochondrial oxalacetate together with decreased rates of oxygen consumption. The further addition of ADP caused a marked increase in oxalacetate, which was associated with the nearly complete cessation of oxygen consumption. In contrast, ATP produced a large reduction in the steady state levels of oxalacetate and increased oxygen consumption. Pyruvate further lowered the level of intramitochondrial oxalacetate and increased oxygen uptake. This suggests that the rate of oxalacetate formation by P-enolpyruvate carboxykinase is dependent on the relative adenine nucleotide balance and that at some critical level of oxalacetate, the formation of NADH for the electron transport chain via malate dehydrogenase becomes minimal. As a consequence, a relationship must exist between the rate of forward flow through malate dehydrogenase and the flow of carbon from oxalacetate to P-enolpyruvate.

It is also evident that rapid rates of oxalacetate formation by
P-enolpyruvate carboxykinase may occur experimentally only when the ratio of ATP:ADP is extremely low. This supports earlier data showing a significant reversal of the enzyme when this same low ratio of ATP:ADP was produced (23). In control experiments with guinea pig liver mitochondria oxidizing malate (Table IV), oligomycin increased oxygen consumption in State 4, and lowered oxalacetate levels. ADP addition decreased oxygen consumption and increased oxalacetate, whereas ATP reversed these effects. Pyruvate further decreased oxalacetate while an increased rate of oxygen consumption was noted, suggesting that NADH availability to the electron transport chain via malate dehydrogenase in State 4 can be controlled by factors regulating the rate and direction of P-enolpyruvate carboxykinase such as the steady state adenine nucleotide balance. In Fig. 8, the inhibition of respiration as a function of P-enolpyruvate concentration was determined at two different levels of malate in both State 3 and State 4. The amount of P-enolpyruvate which produced a 50% inhibition in State 3 with 10 mm malate was 3.1 mm. This value is reduced to 1.7 mm when malate was reduced to 2.5 mm. The effects of P-enolpyruvate on State 4 respiration were slight, supporting the hypothesis that high levels of ADP are required for significant rates of P-enolpyruvate utilization (22).

**DISCUSSION**

The intramitochondrial localization of P-enolpyruvate carboxykinase considerably complicates an understanding of the control of oxalacetate metabolism in most animal species. Since oxalacetate is the common point of overlap between the pathways of gluconeogenesis, lipogenesis, and energy production by the citric acid cycle, controls on the over-all utilization and metabolic flux of this intermediate must be vital to any regulatory considerations of the individual pathways involved. In this study, the controlling influence on the concentration and utilization of oxalacetate have been evaluated in isolated, intact mitochondria.

**NAD⁺-Malate Dehydrogenase Equilibrium**—Previous investigations on mitochondrial oxalacetate metabolism have assumed that the steady state level of this intermediate was determined by a malate dehydrogenase equilibrium and its interactions with the free pools of the nicotinamide coenzymes and the intramitochondrial level of malate (6, 8, 24, 25). The principal experimental support for such an assumption has been the high in vitro activity of NAD⁺-malate dehydrogenase in mitochondria from guinea pig (6) and rat liver (8). Computer simulations of hepatic metabolism have yielded differing conclusions as to the existence of an equilibrium state of the enzyme in vivo (26, 27). In the study finding a nonequilibrium state (27), the conclusion was derived from a discrepancy of only about 40% between the calculated and measured oxalacetate levels in the whole tissue. The results of this study show clearly that NAD⁺-malate dehydrogenase functions in an apparent equilibrium state in intact liver mitochondria, and that other metabolic pathways and

**Table III**

*Effects of adenine nucleotides upon P-enolpyruvate inhibition of malate respiration*

Guinea pig liver mitochondrial suspensions oxidizing malate (10 mm) in the oxygen cuvette were sampled at the points indicated in Fig. 7. Oligomycin additions were titrated for each mitochondrial preparation. The mean amount used was 1.92 μg of oligomycin per mg of mitochondrial protein. Pyruvate was added to a final concentration of 10 mm, while ADP addition was 250 μg, and ATP addition was 1 mm. P-enolpyruvate was added to a final level of 5 mm. Samples were rapidly removed, acidified, and oxalacetate determined on the resulting neutralized extracts (see "Methods"). Separate samples of the mitochondrial suspension were saved for nitrogen determination. Mitochondrial matrix volume was taken from the data of Pfaff et al. (44). The data are given as nanomoles per mg of mitochondrial protein or nitrogen, and are the means ± standard error for at least three experiments.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Addition</th>
<th>Oxygen consumption</th>
<th>Oxalacetate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>μg/s/mg protein</td>
<td>mmole/mg nitrogen</td>
</tr>
<tr>
<td>A</td>
<td>Oligomycin</td>
<td>0.066 ± 0.010</td>
<td>0.069 ± 0.009</td>
</tr>
<tr>
<td>B</td>
<td>P-enolpyruvate</td>
<td>0.031 ± 0.005</td>
<td>0.052 ± 0.001</td>
</tr>
<tr>
<td>C</td>
<td>ADP</td>
<td>0.000 ± 0.000</td>
<td>0.075 ± 0.008</td>
</tr>
<tr>
<td>D</td>
<td>ATP</td>
<td>0.049 ± 0.008</td>
<td>0.133 ± 0.024</td>
</tr>
<tr>
<td>E</td>
<td>Pyruvate</td>
<td>0.193 ± 0.017</td>
<td>0.060 ± 0.007</td>
</tr>
</tbody>
</table>

**Table IV**

*Influence of adenine nucleotides upon oxalacetate levels during State 4 respiration from malate*

Guinea pig liver mitochondrial suspensions oxidizing malate (10 mm) in the oxygen cuvette were sampled at various points indicated below. Samples were rapidly removed, acidified, and oxalacetate determined on the resulting neutralized extracts (see "Methods"). Oligomycin addition was titrated for each mitochondrial preparation. The mean amount used was 1.86 mg of oligomycin per mg of mitochondrial protein. Pyruvate was added to a final concentration of 10 mm where indicated. Other details as in Table I and "Methods."

<table>
<thead>
<tr>
<th>Respiratory state</th>
<th>Addition</th>
<th>Oxygen consumption</th>
<th>Oxalacetate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>μg/s/mg protein</td>
<td>mmole/mg nitrogen</td>
</tr>
<tr>
<td>State 3</td>
<td>Oligomycin</td>
<td>0.050 ± 0.007</td>
<td>0.101 ± 0.014</td>
</tr>
<tr>
<td>State 4</td>
<td>ADP</td>
<td>0.048 ± 0.000</td>
<td>0.109 ± 0.011</td>
</tr>
<tr>
<td>State 4</td>
<td>ATP</td>
<td>0.097 ± 0.014</td>
<td>0.032 ± 0.012</td>
</tr>
<tr>
<td>State 4</td>
<td>Pyruvate</td>
<td>0.114 ± 0.016</td>
<td>0.045 ± 0.008</td>
</tr>
</tbody>
</table>

**Fig. 8.** Oxygen consumption in State 3 (●) and State 4 (○) from malate as a function of P-enolpyruvate concentration. Two concentrations of malate were used, 2.5 mm (---) and 10 mm (—−−). Other details as in Fig. 1 and "Methods."
different physical properties play an important role in influencing the steady state concentrations of oxalacetate in guinea pig and rat liver mitochondria.

Evidence that the NAD$^+$-malate dehydrogenase system reacts functionally in an equilibrium manner is provided by an analysis of the changes in oxalacetate levels noted in Fig. 3 and Table I. Increasing oxidation of the pyridine nucleotides during State 3 was associated with increasing concentrations of oxalacetate. Correspondingly, the transition from State 3 to State 4, as indicated by the reduction of pyridine nucleotides, was associated with a proportional decline in the concentration of oxalacetate in both rat and guinea pig mitochondria. A further reduction in oxalacetate following pyruvate or glutamate addition in State 4 was accompanied by a still greater reduction of the pyridine nucleotides, which was proportional to the decrease in oxalacetate. The fluorescence determination of pyridine nucleotide oxidation-reduction state used in this study does not clearly discriminate between the free and bound nucleotide pools, although the rapid changes may involve predominantly the bound pools (28-30). However, similar changes in the intramitochondrial oxidation-reduction potential have been noted previously for the free pools of NAD$^+$ and NADH$^+$ as calculated from the components of the $\beta$-hydroxybutyrate dehydrogenase equilibrium (7, 23). These results indicate that the NAD$^+$-malate dehydrogenase system reacts functionally as if in an equilibrium state. Whether or not a true chemical equilibrium exists can be evaluated by a calculation of the equilibrium constant of the enzyme system under a variety of conditions. Using the ratios of free NAD$^+$ to free NADH (7), the intramitochondrial concentration of malate (10), together with the data of Table I for oxalacetate levels permits an approximation of the apparent $K_{eq}$ for the intact mitochondrial system. The equilibrium constant of the mitochondrial system in State 4 was calculated as $2.42 \times 10^{-5}$, early in State 3 as $2.10 \times 10^{-5}$, and late in State 3 as $7.2 \times 10^{-5}$. These should be compared to the $K_{eq}$ of the isolated enzyme of $2.78 \times 10^{-5}$ (8). It seems probable then that NAD$^+$-malate dehydrogenase in the intact mitochondria is essentially at an equilibrium point although some tendency toward disequilibrium may occur during the marked inhibition of late State 3 respiration from malate.

It is clear from the results that if NAD$^+$-malate dehydrogenase functions at equilibrium, forward flux through this enzyme must be influenced by equilibrium kinetics. Increased oxalacetate levels are associated with decreased rates of malate oxidation resulting from a decreased formation of NADH particularly in mitochondria from guinea pig. This then accounts for the nonlinear and terminally inhibited respiration observed with malate alone as a substrate. Reversal of P-enolpyruvate carboxykinase with added P-enolpyruvate, bicarbonate, and ADP provides a mechanism to generate and vary the intramitochondrial concentration of oxalacetate. Our method of varying oxalacetate concentration by direct intramitochondrial generation yields more easily interpretable results than previous studies which used large quantities of oxalacetate added to the medium. This provides a more accurate estimation of the effective inhibitory concentration of oxalacetate because of the limited permeability of the mitochondrial membrane for this intermediate when added externally (31). The results of the present study are similar qualitatively to prior data (32) which showed an oxidation of the respiratory chain-linked pyridine nucleotides by added oxalacetate. However, 3 orders of magnitude difference exists in the estimated concentration of oxalacetate necessary to produce these effects. As a result of large increases in oxalacetate, State 4 respiration effectively ceases (Fig. 7), suggesting that the availability of NADH for the electron transport chain becomes highly limited. This would be the expected result if a true equilibrium state exists between malate, oxalacetate, and the pyridine nucleotides. It is therefore likely that any factor influencing the concentration of oxalacetate must alter reciprocally the steady state levels and the rate of formation of reduced nicotinamide coenzyme for the respiratory chain, and this is observed with the addition of glutamate. These results extend the earlier observations of Schollmeyer and Klingenberg, in rat liver mitochondria (33, 34), showing a correlation between mitochondrial respiration and oxalacetate levels with succinate as substrate. It was also shown that incubation of mitochondria with either pyruvate or glutamate lowered the levels of oxalacetate, and respiration for succinate was facilitated.

P-enolpyruvate Carboxykinase and Control of Oxalacetate Utilization—The large activity of P-enolpyruvate carboxykinase in guinea pig liver mitochondria appears to play an important role in the control of oxalacetate metabolism in this species. Rapid rates of P-enolpyruvate formation within the matrix space must produce a rapid rate of GDP formation and hence, by transphosphorylation, of ADP (23). As a direct result of mitochondrial P-enolpyruvate synthesis, an internal ADP-regenerating system is then established, the over-all rate of which is dependent upon both oxalacetate and ATP availability within the mitochondria. This regeneration may then account for the lower ADP to oxygen ratios, the persistent oxidation of the nicotinamide coenzymes during the inhibited transition from State 3 to State 4, the prolongation of State 3 respiration, and the increased rates of oxygen consumption in State 4 observed in guinea pig mitochondria. It seems probable that the rate of oxalacetate removal by P-enolpyruvate carboxykinase represents the principal limitation to the rate of forward flux through NAD$^+$-malate dehydrogenase. In State 3 respiration, when the availability of ATP relative to that of ADP is reduced, a decreased rate of GTP formation by transphosphorylation will occur, thereby reducing the maximum possible rate of P-enolpyruvate synthesis (23). It is not unexpected that the over-all effect of such a mechanism is a considerable prolongation of the State 3 to State 4 transition associated with highly oxidized pyridine nucleotides. That this occurs to a much lesser extent in rat liver supports this conclusion since the greatly decreased rate of P-enolpyruvate formation in this species results in little or no ADP regeneration. Consequently, only a slight inhibition of respiration can be detected during the transition from State 3 to State 4 in the rat. Glutamate, and to a lesser extent pyruvate, are capable of decreasing the inhibition of NAD$^+$-malate dehydrogenase in the guinea pig by providing alternate mechanisms for the utilization of oxalacetate other than P-enolpyruvate carboxykinase. The effect of glutamate addition appears mediated by aspartate aminotransferase rather than by glutamate dehydrogenase since the effect may be blocked by the prior addition of aminoxyacetate, a known inhibitor of the former enzyme (35, 36). Both glutamate and pyruvate serve to reduce the intramitochondrial concentration of oxalacetate, producing a decreased rate of P-enolpyruvate formation and hence, ADP regeneration. The decrease in ADP regeneration then results in an increase in the P:O ratios, an increase in the State 3 rate of respiration, and a decrease in the rate of oxygen consumption in State 4. Pyridine nucleotides remain relatively reduced as a reciprocal change to the decreased oxalacetate levels.

1 Alan J. Garber, unpublished results.
Other metabolic consequences arise from the intramitochondrial formation of P-enolpyruvate. Since the synthesis of P-enolpyruvate involves a decarboxylation, a net production of protons results within the matrix space. Translocation of these protons may account for the reductions in $H^+:ADP$ ratios during State 3, and for the persistent, oligomycin-insensitive acidification noted during State 4 in the mitochondrial suspensions from guinea pig liver. Factors serving to decrease the rate of P-enolpyruvate formation such as glutamate addition decrease the rate of acidification and restore ratios of $H^+:ADP$ toward normal values. Little or no acidification in State 4 was observed in suspensions of rat liver mitochondria, and no changes produced by glutamate were observed in $H^+:ADP$ ratios supporting the concept that the acidification results from P-enolpyruvate formation. It is therefore likely that many of the observed kinetic differences between guinea pig and rat liver mitochondria result from and depend upon rapid rates of P-enolpyruvate formation by guinea pig liver mitochondria.

It should be noted that some critical concentration of oxalacetate must be exceeded before the inhibitions to mitochondrial function resulting from P-enolpyruvate synthesis become evident. In previous studies we have shown that the level of oxalacetate as mediated by the oxidation-reduction potential regulated in part, the rate of P-enolpyruvate formation (6, 22). This result is obtained if the concentration of oxalacetate is at or below the Michaelis constant of the enzyme. In State 3, when oxalacetate levels in the presence of glutamate increase to approximately 0.5 µM, little effect of P-enolpyruvate formation on mitochondrial function may be observed because the rate of P-enolpyruvate synthesis is reduced from 7.26 to 1.24 nmoles per min per mg of protein in the presence of glutamate. However, in the presence of pyruvate, oxalacetate concentrations ranged between 15 and 35 µM, causing significant alterations in oxygen consumption, pyridine nucleotide reduction, and proton translocations. At this time the rate of P-enolpyruvate formation was found to be 5.63 nmoles per min per mg of protein. It follows then, that the "$K_m$" of the pathway for P-enolpyruvate synthesis may also be in that same range of oxalacetate concentrations. Kinetic data on the isolated guinea pig liver enzyme have yielded a $K_m$ for oxalacetate of 1 to 2 $\times 10^{-3}$ M (37, 38). The lower apparent $K_m$ for mitochondrial P-enolpyruvate formation suggests either that some modification of enzyme kinetics occurs in the intact mitochondria, or that most of the oxalacetate is highly compartmented in the vicinity of P-enolpyruvate carboxykinase. An alternative and more likely explanation is that the Michaelis constant of the enzyme for oxalacetate is considerably lower than previously suspected. Recent work on the rat liver cytosolic form of P-enolpyruvate carboxykinase using an equilibrium displacement assay with malate dehydrogenase (39) has found a $K_m$ in the range of 9 to 30 µM, rather than the much higher values obtained previously (40). A redetermination of the Michaelis constants for the guinea pig liver enzyme using this same technique seems in order. However, it appears likely that the true $K_m$ for oxalacetate must be in that same range as that for the rat liver enzyme. As a consequence, it appears that the increased levels of oxalacetate are initially necessary to increase the rate of P-enolpyruvate formation in the mitochondria. After this, oxalacetate tends to remain elevated owing to the process of ATP regeneration, resulting from P-enolpyruvate formation. Thus, once initiated, P-enolpyruvate formation tends to be self-perpetuating by leading to oxidized pyridine nucleotides and high levels of oxalacetate.

Several alternative explanations for the inhibition of malate oxidation in guinea pig liver but not in rat liver mitochondria cannot be entirely excluded at this time. One possible explanation is that the increased concentrations of oxalacetate may be more inhibitory to the NAD$^+$-malate dehydrogenase of guinea pig than that of rat liver. The maximum levels of oxalacetate found in this study are substantially less (50 to 100 µM) than the described $K_m$ for the enzyme (0.25 mM) (41). However, significant compartmentation of the malate dehydrogenase system may exist, resulting in a similar compartmentation for oxalacetate. Evidence has been forwarded for compartmentation of oxalacetate with reference to succinate dehydrogenase (42), and any change in the degree of compartmentation could increase oxalacetate concentrations many fold in the vicinity of a malate dehydrogenase-P-enolpyruvate carboxykinase functional unit. It seems more likely that rat liver mitochondria may have a greater supply of endogenous substrates which facilitate the removal of oxalacetate than do guinea pig liver mitochondria. The well washed mitochondria of both species contain no detectable glutamate or pyruvate, although sources of acetyl-CoA such as endogenous fatty acids have not been evaluated. Finally, it is possible that the accumulation of intramitochondrial oxalacetate competitively diminishes the permeability of the mitochondrial membrane for external malate in guinea pig mitochondria (43). However, oxalacetate does not appear to compete with the malate transport mechanism.

Control of Oxalacetate Metabolism for Gluconeogenesis—These in vitro observations on the metabolic behavior of isolated guinea pig and rat liver mitochondria remarkably reflect certain dissimilarities noted in vivo between these two species. During the increased gluconeogenesis observed in the fasted state (4), a marked shift toward oxidation of the mitochondrial nicotinamide coenzymes was found in species containing a significant mitochondrial activity of P-enolpyruvate carboxykinase (6, 7). In striking contrast, the rat shows a shift toward reduction upon fasting (8). In view of the differences in metabolism between species in this study it seems likely that the shift toward oxidation may, in fact, result from the metabolic consequences of the intramitochondrial localization of P-enolpyruvate carboxykinase. Presumably, this results from the intramitochondrial regeneration of ADP which accompanies mitochondrial P-enolpyruvate formation, and causes an increased level of oxalacetate and of pyridine nucleotide oxidation. Support for this conclusion is provided by studies of freeze-clamped livers of fed and fasted guinea pigs and rabbits showing that the steady state hepatic concentration of glutamate decreases in the fasted state, while it increases in the rat (6, 8). This alteration provides a mechanism for the differential shifts of mitochondrial pyridine nucleotides and oxalacetate levels as noted above. In addition, since gluconeogenesis is an energy-requiring process using ATP and forming ADP, an increased level of ADP would serve to intensify the magnitude of any oxidation-reduction potential change in the guinea pig. This occurs independently of the results of substrate interplay on the ratio of NAD$^+$ to NADH. These results may then account for the observation that a calculation of the mitochondrial oxalacetate levels in vivo on fasting, shows an 8-fold increase in the guinea pig, while the rat shows a 4-fold reduction (6). We may then conclude that the shift toward oxidation noted in livers of fasted guinea pigs and rabbits derives from, and reflects, the intrinsic mechanism of gluconeogenesis in species containing a mitochondrial activity of P-enolpyruvate carboxykinase.

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Alan J. Garber and Leon Salganicoff


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