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Synthesis of malate from phospho*enol*pyruvate by rabbit liver mitochondria: implications for lipogenesis

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(1) Rabbit liver mitochondria can convert exogenous phospho*enol*pyruvate to malate. (2) Malate production is dependent on phospho*enol*pyruvate and HCO_3^- and is stimulated by CN^- or malonate alone and especially in combination. (3) Malate production is inhibited 70% by 3-mercaptopicolinate, a specific inhibitor of phospho*enol*pyruvate carboxykinase, and 50–60% by 1,2,3-benzenetricarboxylate, an inhibitor of the tricarboxylate transporter. (4) Rat liver mitochondria incubated with phospho*enol*pyruvate under identical conditions do not produce malate. (5) Malate production from phospho*enol*pyruvate is stimulated by exogenous GDP or IDP but not by ADP. (6) Data support the conclusion that malate is being produced from oxalacetate generated by reversal of mitochondrial phospho*enol*pyruvate carboxykinase. A possible role for this enzyme in hepatic lipogenesis is suggested.

Introduction

The intracellular distribution of hepatic phospho*enol*pyruvate carboxykinase (GTP : oxaloacetate carboxy-lyase (transphosphorylating), EC 4.1.1.32) varies among species [1–3]. This enzyme activity is located mainly in cytoplasm of rodent liver and mitochondria of avian liver, whereas significant amounts are found in both of these compartments in hepatocytes of most other species including rabbit, guinea pig and human.

Cytoplasmic *P-enol*pyruvate carboxykinase is inducible by conditions conducive to gluconeogenesis [4–7] and is considered obligatory for maximal rates of hepatic gluconeogenesis in any species which possesses it. In contrast, mitochondrial *P-enol*pyruvate carboxykinase is constitutive and unresponsive to metabolic or hormonal alterations. Nonetheless the fact that direct (quinolinate) or indirect (aminooxyacetate) inhibitors of carbon flow through cytoplasmic *P-enol*pyruvate carboxykinase do not decrease gluconeogenesis at all in pigeon liver and by only 50–60% in fasted guinea pig and rabbit livers suggests that mitochondrial *P-enol*pyruvate carboxykinase is also a major contributor to glucose formation, especially from lactate [8–10].

Recent work suggests that the *P-enol*pyruvate carboxykinase-mediated reaction may be near equilibrium under conditions which may be at-

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Abbreviations: *P-enol*pyruvate carboxykinase, phospho*enol*-pyruvate carboxykinase; *P-enol*pyruvate, phospho*enol*pyruvate; Mops, morpholinepropanesulfonic acid.

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tained in vivo. For example, Rognstad finds considerable $^{14}CO_2$ incorporation into glucose being formed from glutamine by rat hepatocytes under conditions where pyruvate carboxylase activity is suppressed [11–13]. Reversibility of the reaction in isolated mitochondria was implicit in recent work of Wilson et al. [14] on intra- and extra-mitochondrial ATP/ADP ratios and in earlier work by Garber and Salganicoff [15] and Garber and Ballard [16]. Söling argues from metabolite concentrations in mitochondria that the net flux through *P-enol*pyruvate carboxykinase could easily be reversed [17].

If the *P-enol*pyruvate carboxykinase reaction in mitochondria were reversed, resulting in a net formation of oxalacetate from extra-mitochondrially produced *P-enol*pyruvate, one could envision a role for the mitochondrial isozyme in lipogenesis. The oxalacetate produced in mitochondria could be used to combine with acetyl CoA to form citrate for export to cytoplasm where lipogenesis could occur with regeneration of pyruvate which could recycle into mitochondria to continually supply acetyl CoA. Some advantages of mitochondrial *P-enol*pyruvate carboxykinase-mediated generation of oxalacetate rather than or in addition to the currently accepted combined reactions of cytoplasmic pyruvate kinase and mitochondrial pyruvate carboxylase include the formation of one equivalent of high-energy phosphate (GDP to GTP) and the electroneutral exchange of mitochondrial citrate for cytoplasmic *P-enol*pyruvate via the tricarboxylate transporter. Perhaps of significance too is the apparent consistency between the degree of hepatic (as opposed to adipose) fatty acid synthesis and the presence of mitochondrial *P-enol*pyruvate carboxykinase activity in various species [18–22].

Accordingly we suggest that mitochondrial *P-enol*pyruvate carboxykinase may participate in hepatic lipogenesis. We report in this paper studies of malate formation from *P-enol*pyruvate by rabbit liver mitochondria and the influences of malonate, CN^-, and various nucleotides on that process.

Materials and Methods

Animals. Male, New Zealand rabbits were obtained from the Animal Resource Facility of the University of North Dakota. Sprague-Dawley rats were obtained from Biolabs, St. Paul, MN. Animals were maintained on tap water and Purina Rabbit or Rodent Chow, respectively. All animals were fasted for 24 h prior to killing and removal of livers for preparation of mitochondria.

Preparation of mitochondria. After excision (and removal of the gall bladder in the case of rabbit livers), livers were ground in a hand-operated household grinder, homogenized in ice-cold 0.25 M sucrose in a Potter-Elvehjem homogenizer and then diluted with 0.25 M sucrose to a volume four times the fresh liver weight. Mitochondria were isolated by differential centrifugation in a refrigerated centrifuge according to the method of Johnson and Lardy [23], washed three times and then dispersed in 0.25 M sucrose (0.5 ml/g fresh liver) using a small hand-operated Potter-Elvehjem homogenizer.

Incubations. Nalgene flasks containing 2.0 ml media and closed with rubber stoppers were pre-incubated for 5 min prior to initiation of experiments by addition of 0.5 ml of mitochondria. Reactions were carried out in a shaker-incubator at 37 °C for the prescribed time and terminated by addition of 1 ml of 15% $HClO_4$.

Incubation media. The basic incubation medium contained 100 mM Mops buffer (pH 7.1), 3.35 mM *P-enol*pyruvate, 20 mM $NaHCO_3$, 10 mM DL-3-hydroxybutyrate, 0.1 mM $MnCl_2$, 7.5 mM $MgSO_4$, 0.25 M sucrose and 0.16 mM NADH plus 3.2 units/ml lactate dehydrogenase. Inclusion of 3-hydroxybutyrate provided NADH for the conversion of *P-enol*pyruvate-derived oxalacetate to malate, since malate is more stable and easier to measure. Lactate dehydrogenase and NADH were added to convert any pyruvate derived from *P-enol*pyruvate by contaminant pyruvate kinase to lactate.

Metabolite assays. The deproteinized reaction media were neutralized to pH 6.5–7 with 5 M K_2CO_3, filtered and assayed for malate immediately or stored overnight at −20 °C prior to assay. Malate was measured by a standard enzymatic method [24].

Protein. Mitochondrial protein was determined using the biuret method as modified by Lee and Lardy [25].

Analysis of enzyme activities. Mitochondria were

freeze-thawed three times and the supernates obtained after centrifugation were assayed for *P-enol*pyruvate carboxykinase at 30 °C and pH 6.8 in 1.0 ml of reaction medium containing 110 mM imidazole-chloride, 45 mM $NaHCO_3$, 2 mM *P-enol*pyruvate, 2 mM $MnCl_2$, 0.8 mM dithiothreitol, 0.5 mM dGDP, 0.15 mM NADH, and 6 units of malate dehydrogenase. The use of dGDP rather than GDP or IDP is routine in our laboratory in order to minimize interference by pyruvate kinase [26]. Pyruvate kinase activity was assayed according to the method of Carminatti [27] as modified by Blair et al. [28].

Antibody inhibition studies. Polyclonal antibodies to mitochondrial and cytoplasmic *P-enol*pyruvate carboxykinase isozymes were generated in pygmy goats according to the method of Dreesman et al. [29]. Polyclonal antibodies were partially purified prior to use and possessed titers greater than 1/2048 against their respective isozymes.

Chemicals. All chemicals used were of the highest grade commercially available and solutions were prepared in glass-distilled water.

Results

Composition of basic system

Preliminary experiments led us to choose a basic incubation medium containing 100 mM Mops buffer (pH 7.1), 7.5 mM $MgSO_4$, 0.1 mM $MnCl_2$, 10 mM DL-3-hydroxybutyrate, 3.35 mM *P-enol*pyruvate, 20 mM $NaHCO_3$, 0.25 M sucrose, 0.16 mM NADH and lactate dehydrogenase (3.2 units/ml). These concentrations of *P-enol*pyruvate, $NaHCO_3$ and 3-hydroxybutyrate were optimal for a linear production of malate over a 10–15 min period of incubation with the amount of mitochondria used (average of about 6 mg protein in 0.25 M sucrose). Malate production by this basic system was nearly totally dependent on *P-enol*pyruvate. Nonetheless, the amounts of malate formation reported in the text and tables are corrected for malate production by appropriate controls incubated in the absence of *P-enol*pyruvate. Malate production by this basic system was also strongly dependent on exogenous HCO_3^- and somewhat stimulated by exogenous Mg^{2+} or Mn^{2+}, although either one sufficed equally well.

TABLE I

MALATE FORMATION FROM *P-ENOL*PYRUVATE IN THE ABSENCE AND PRESENCE OF NaCN AND/OR MALONATE AND THE INFLUENCE OF GDP THEREON

Mitochondria were incubated at 37 °C for 15 min in a basic medium containing 100 mM Mops buffer (pH 7.1), 3.35 mM *P-enol*pyruvate, 20 mM $NaHCO_3$, 7.5 mM $MgSO_4$, 0.1 mM $MnCl_2$, 10 mM DL-3-hydroxybutyrate, 0.25 M sucrose and 0.16 mM NADH plus lactate dehydrogenase (3.2 units/ml). Other additions included 1 mM NaCN and 10 mM malonate as indicated. Data represent the results ± S.D. from three identical experiments, using the average of duplicate observations in each case.

	Malate (nmol/15 min per mg protein)			
	no addition	+ CN^-	+ malonate	+ CN^- + malonate
Basic	19 ± 5	41 ± 7	48 ± 6	64 ± 7
Basic + 0.5 mM GDP	50 ± 2	77 ± 17	91 ± 28	105 ± 5

Influence of CN^- and/or malonate on malate formation

Data in Table I show that the system just described produced 19 nmol malate/15 min per mg protein. The inclusion of CN^- increased malate production to 41 nmol, presumably by inhibiting electron transport (thus increasing the NADH/NAD^+ ratio and facilitating the reduction of oxalacetate to malate). Inclusion of malonate increased malate production to 48 nmol, presumably by inhibiting succinate dehydrogenase-catalyzed equilibration of malate with succinate. Addition of both CN^- and malonate increased malate production still further to 64 nmol. Other data in Table I show that inclusion of 0.5 mM GDP in the basic system increased malate formation about 2-fold above the amount formed in its absence. Addition of GDP with CN^- or malonate alone or in combination also increased malate formation 1.5–2-fold.

Source of malate being formed

As mentioned earlier, the malate being formed in the system described is dependent on the presence of *P-enol*pyruvate and HCO_3^-. However, the possibility existed that *P-enol*pyruvate was first being converted by contaminant pyruvate kinase to pyruvate which was then carboxylated by

pyruvate carboxylase to form oxalacetate. The assayable activity of pyruvate kinase in several preparations of mitochondria was less than 10% that of *P-enol*pyruvate carboxykinase when both were assayed under optimal conditions (data not presented). To preclude a significant contribution by pyruvate kinase to malate production by our system, we did not add any exogenous K^+, since pyruvate kinase is known to be K^+-dependent. We also included lactate dehydrogenase and NADH to reduce conversion of any *P-enol*pyruvate-derived pyruvate to lactate.

However, to test directly the possibility that malate was being produced from pyruvate derived from *P-enol*pyruvate, we substituted 6.7 mM pyruvate for 3.35 mM *P-enol*pyruvate in our system and observed no significant formation of malate. We also determined that 500 μM 3-mercaptopicolinate (a specific inhibitor of *P-enol*pyruvate carboxykinase [30–35]) inhibits malate formation from *P-enol*pyruvate by 70% in either the absence or presence of GDP (see Table II).

The possibility also existed that malate was being generated by contaminant cytoplasmic isozymes of *P-enol*pyruvate carboxykinase and malate dehydrogenase rather than the mitochondrial isozymes. However we found that 15 mM 1,2,3-benzenetricarboxylate (an inhibitor of the tricarboxylate transporter which transports *P-enol*pyruvate across the mitochondrial membrane [36–41]) inhibits malate production by about 50 and 60% in the absence and presence of GDP (see Table II). Furthermore, partially purified polyclonal antibodies to mitochondrial *P-enol*pyruvate carboxykinase immunoprecipitated 92–97% of the total *P-enol*pyruvate carboxykinase activity contained in seven different preparations of lysed mitochondria whereas antibodies to cytoplasmic *P-enol*pyruvate carboxykinase immunoprecipitated from 0 to 17% of the total activity in the same preparations (see Table III).

Since rat liver contains primarily cytoplasmic *P-enol*pyruvate carboxykinase, a lack of malate production by isolated rat liver mitochondria would provide additional evidence for the participation of mitochondrial *P-enol*pyruvate carboxykinase in malate production by rabbit liver mitochondria. Rat liver mitochondria incubated with *P-enol*pyruvate in either the absence or the presence of GDP produced negligible quantities of malate compared to the amounts generated by rabbit liver mitochondria.

The aforementioned experimental observations lead us to conclude that the malate being produced by our system is from oxalacetate derived primarily by reversal of mitochondrial *P-enol*pyruvate carboxykinase.

TABLE II

INHIBITION OF MALATE FORMATION FROM *P-ENOL*PYRUVATE BY 3-MERCAPTOPICOLINATE OR 1,2,3-BENZENETRICARBOXYLATE AND THE INFLUENCE OF GDP THEREON

Mitochondria were incubated as described in Table I and other additions included 500 μM 3-mercaptopicolinate (3-MP) and 15 mM 1,2,3-benzenetricarboxylate (BTCA). Data represent results ± S.D. from three identical experiments, using the average of duplicate observations in each case.

	Malate (nmol/15 min per mg protein)		
	no addition	+3-MP	+BTCA
Basic + CN^- + malonate	64 ± 7	18 ± 4	34 ± 6
Basic + CN^+ + malonate + 0.5 mM GDP	105 ± 5	35 ± 7	42 ± 11

Effects of nucleotides on malate production

Since GDP (or IDP) is a required cofactor for reversal of the *P-enol*pyruvate carboxykinase reaction, we examined the influences of exogenous nucleotides on malate production. Data indicated that malate production from *P-enol*pyruvate is significantly increased by either 0.5 mM GDP (1.65-fold control, $n = 3$, $P < 0.02$) or IDP (1.56-fold control, $n = 3$, $P < 0.025$). Conversely, ADP had no stimulatory influence on malate production. The stimulatory effects of GDP and IDP were not anticipated since these nucleotides are not thought to cross the inner mitochondrial membrane.

The effect of exogenous GDP could have been due to leakage of some mitochondrial *P-enol*pyruvate carboxykinase and malate dehydrogenase which could, with GDP, form extramitochondrial oxalacetate which could subsequently be reduced to malate in the presence of exogenous NADH added as part of the pyruvate

TABLE III

ANTIGEN-ANTIBODY INHIBITION STUDIES USING POLYCLONAL ANTIBODIES AGAINST MITOCHONDRIAL AND CYTOPLASMIC *P-ENOL*PYRUVATE CARBOXYKINASES (PEPCK)

Aliquots of high-speed supernates prepared from three-times freeze-thawed mitochondria were incubated with antisera (1–3-fold the amount estimated to cause 100% precipitation of the appropriate enzyme antigen) for 60 min in a shaker-incubator at 37 °C, centrifuged, and aliquots of the resulting supernates were assayed for *P-enol*pyruvate carboxykinase as described in the methods section.

No. expts.	Percent PEPCK activity						
	control	antisera (mg protein) to mitochondrial PEPCK			antisera (mg protein) to cytoplasmic PEPCK		
		7.1	14.3	21.4	7.1	14.3	21.4
7	100	8.81 ± 3.9	3.26 ± 1.6	3.25 ± 0.8	114 ± 10	99.8 ± 15	83.4 ± 24

TABLE IV

INFLUENCE OF EXOGENOUS GDP OR NADH ON MALATE PRODUCTION BY INTACT RABBIT LIVER MITOCHONDRIA

The reaction medium contained 3.35 mM *P-enol*pyruvate, 20 mM $NaHCO_3$, 100 mM Mops buffer (pH 7.1), 7.5 mM $MgSO_4$, 0.1 mM $MnCl_2$, 10 mM DL-3-hydroxybutyrate, 10 mM malonate, 1 mM NaCN, and 0.25 M sucrose. Data represent the average of duplicate observations in each case.

State of mitochondria	Malate (nmol/15 min per mg protein)			
	zero-time control	basic	0.5 mM GDP	0.16 mM NADH
Intact (Expt. 1)	3	59	112	62
Intact (Expt. 2)	2	24	38	28

trap. To test this possibility, malate production was measured in the absence and presence of exogenous GDP or NADH. Data in Table IV show that malate production was not stimulated by exogenous NADH whereas exogenous GDP caused a considerable increase in malate production. These data suggest that exogenous GDP and IDP are stimulating malate production primarily by mitochondrial *P-enol*pyruvate carboxykinase contained in intact mitochondria.

Discussion

Cytoplasmic *P-enol*pyruvate carboxykinase has an essential role in hepatic gluconeogenesis from non-carbohydrate sources. Conversely, mitochondrial *P-enol*pyruvate carboxykinase, at least in birds where it is the only isozyme of consequence, appears to participate in glucose formation only by recycling lactate to glucose via the Cori cycle [42]. Since lactate carbon can obviously be converted to glucose via cytoplasmic *P-enol*pyruvate carboxykinase, the mitochondrial isozyme would seem to have no essential role in species possessing significant amounts of both isozymes. The question arises then as to whether mitochondrial *P-enol*pyruvate carboxykinase plays a role in some pathway other than formation of *P-enol*pyruvate en route to glucose. Might mitochondrial *P-enol*pyruvate carboxykinase run in the reverse direction to catabolize rather than to synthesize *P-enol*pyruvate?

A variety of evidence suggests that oxalacetate formation by reversal of the *P-enol*pyruvate carboxykinase-mediated reaction is feasible. The apparent K_{eq} of the enzyme indicates that the reaction is readily reversible [17]. The activity of the enzyme is conveniently assayed 'in vitro' in the reverse direction by measuring fixation of labeled CO_2 [43], the production of ITP (or GTP) [44], or NADH disappearance due to reduction of the oxalacetate formed to malate by malate dehydrogenase [43]. In certain invertebrates such as helminths [45] and marine bivalves [46], a fermentation of glucose to succinate occurs under anaerobic conditions, and involves a routing of *P-enol*pyruvate to oxalacetate via *P-enol*pyruvate carboxykinase. Söling has predicted that the net flux of the mitochondrial isozyme could go in the direction of oxalacetate formation [17]. Wilson et al. [14] utilized the reversibility of the enzyme in mitochondria from pigeon and guinea pig livers to

evaluate the intra- and extra-mitochondrial ATP/ADP ratios. Garber and Salganicoff [15] demonstrated reversal of *P-enol*pyruvate carboxykinase in guinea pig liver mitochondria when the ATP/ADP ratio was low, and Garber and Ballard [16] suggested that reversal of the mitochondrial isozyme may provide a reserve of mitochondrial oxalacetate when the ATP/ADP ratio falls or when oxalacetate formation by the tricarboxylic acid cycle is prevented by a high $NADH/NAD^+$ ratio.

We have shown directly the ability of *P-enol*pyruvate carboxykinase in mitochondria isolated from rabbit liver to convert exogenous *P-enol*pyruvate to oxalacetate. Conditions used to assure conversion of any oxalacetate so generated to malate included addition of exogenous 3-hydroxybutyrate and CN^-, both of which would help to ensure a high $NADH/NAD^+$ ratio. Equilibration of malate with succinate was precluded by addition of malonate. In this system, malate production was increased linearly about 4-fold by increasing the concentration of DL-3-hydroxybutyrate from 0 to 10 mM (data not shown). In the presence of 10 mM DL-3-hydroxybutyrate, 1 mM CN^- and 10 mM malonate increased malate formation 2- and 2.5-fold, respectively, when added alone and about 3.5-fold when added together (Table I).

Since pyruvate could not replace *P-enol*pyruvate as a substrate for malate production by this system and since formation of malate from *P-enol*pyruvate was decreased 70% by 3-mercaptopicolinate (Table II), which is a specific inhibitor of *P-enol*pyruvate carboxykinase in both the forward and reverse directions [34,35] and is capable of crossing the mitochondrial membrane [31], malate production from *P-enol*pyruvate must certainly be occurring by reversal of *P-enol*pyruvate carboxykinase.

Furthermore, our data suggest that malate formation from *P-enol*pyruvate is being mediated primarily by the mitochondrial isozyme since essentially all of the *P-enol*pyruvate carboxykinase activity in representative samples of our mitochondrial preparations was immunoprecipitated by polyclonal antibodies contained in sera from goats immunized with the mitochondrial isozyme (Table III). Since malate formation from *P-enol*pyruvate was decreased some 50–60% by 1,2,3-benzenetricarboxylate (Table II), which is an inhibitor of the tricarboxylate transporter [36–38], it can be concluded further that most of the malate is being generated from *P-enol*pyruvate subsequent to its internalization into the mitochondrial matrix.

Either GDP or IDP is required for reversal of the *P-enol*pyruvate carboxykinase reaction. Since some malate is being formed from *P-enol*pyruvate in the absence of any exogenous nucleotides (Table I), sufficient endogenous GDP must be available to initiate the reverse reaction and the GTP generated must be re-converted to GDP by reacting with other mono- or diphosphorylated nucleotides. Although exogenous ADP did not stimulate malate production, exogenous IDP and especially GDP increased malate formation from *P-enol*pyruvate 1.6-fold. Since significant quantities of non-adenine nucleotides are not thought to cross the mitochondrial membrane [17], the possibility of mitochondrial damage and release of mitochondrial *P-enol*pyruvate carboxykinase and malate dehydrogenase was considered as a source of malate production (in experiments where exogenous NADH was being added as part of a pyruvate trap). However, exogenous GDP stimulated malate production in the absence of exogenous NADH whereas exogenous NADH had no stimulatory influence on malate formation in the absence of exogenous GDP (Table IV). These data also suggest that malate production is occurring inside intact mitochondria. If so, perhaps small but adequate amounts of GDP or IDP are crossing the mitochondrial membrane to cause their stimulatory effects. Duee and Vignais have shown that small amounts of GDP, CDP and UDP are taken up by mitochondria by an atractyloside-insensitive process [47] and Söling [17] points out that the source of mitochondrial guanine nucleotides is unknown.

If hepatic mitochondrial *P-enol*pyruvate carboxykinase were to catalyze the formation of oxalacetate from *P-enol*pyruvate in vivo, then it could play a significant role in hepatic lipogenesis from carbohydrate sources (see Fig. 1). In fact, oxalacetate formation directly from *P-enol*pyruvate affords some distinct advantages compared to oxalacetate formation from *P-enol*pyruvate by the combined actions of pyruvate kinase and pyruvate

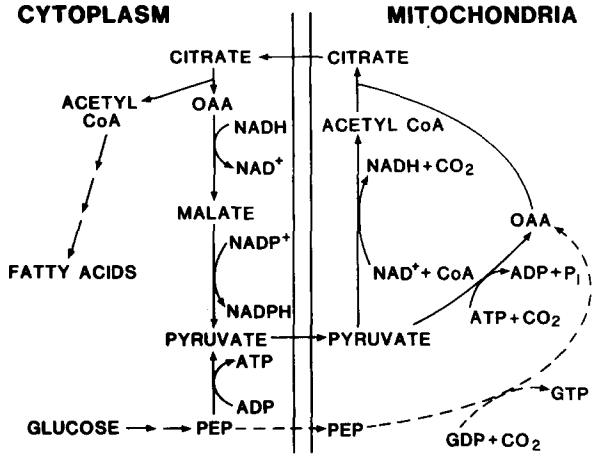

Fig. 1. Alternative pathways for generation of mitochondrial oxalacetate (OAA) from cytoplasmic *P-enol*pyruvate (PEP). The pathway proposed in this study is shown by dashed arrows.

carboxylase. One obvious advantage is the preservation of one equivalent of high-energy phosphate as GDP is phosphorylated to GTP. Still another advantage is the electro-neutral exchange of intra-mitochondrial citrate for extra-mitochondrial *P-enol*pyruvate, and lack of electroneutrality is a major shortcoming of the currently accepted pathway as pointed out by Conover [48].

Is the amount of malate formation in these experiments significant with respect to the quantity of acetyl groups required to support hepatic fatty acid synthesis? Our system forms about 100 nmol malate/15 min per mg mitochondrial protein in the presence of CN^-, malonate and GDP (Table I). Our dilution of mitochondria was such that 6 mg was equivalent to 1 g of liver wet weight (on average). If one assumes that only about half of the mitochondria remain after homogenization, isolation, and washing, then our system was forming about 4.8 μmol malate/h per g of wet weight. Spencer et al. [49] report that high-speed hepatic supernates from fed rats incorporate about 20 μmol citrate into fatty acids/h per g wet weight and Chernick and Chaikoff [50] report rates of 6–12 μmol of acetyl equivalents from glucose incorporated into fatty acids/h per g liver. Thus our system which may not be operating at maximal efficiency is producing malate within a reasonable range of the amount needed to provide citrate (acetyl groups) for these reported rates of fatty acid formation. Since our rates of malate formation from *P-enol*pyruvate appear reasonable, the physiological significance of these observations deserves further investigation.

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